Contribution of toll-like receptor signaling pathways to breast tumorigenesis and treatment

La Creis R Kidd1,*
Erica N Rogers2,*
Susan T Yeyeodu2
Dominique Z Jones1
K Sean Kimbro2
1Department of Pharmacology and Toxicology, University of Louisville, Louisville, KY, 2Biomedical/Biotechnology Research Institute (BBRI), North Carolina Central University, Durham, NC, USA
*These authors contributed equally to this manuscript and are co-first authors

Abstract: Mounting evidence indicates that anomalies in the inflammatory and immune response pathways are essential to tumorigenesis. However, tumor-based innate immunity initiated by transformed breast epithelia tissues has received much less attention. This review summarizes published reports on the role of the toll-like receptor signaling pathway on breast cancer risk, disease progression, survival, and disease recurrence. Specifically, we discuss the underlying biological mechanisms that contribute to the tumorigenic and/or anti-tumorigenic properties of toll-like receptors and their associated agonists in relation to breast tumorigenesis and cancer treatment. Further, we use results from preclinical, clinical, and population-based studies as prompts for the exploration of new and more effective breast cancer therapies. As the knowledge base of innate immunity’s involvement in breast cancer progression increases, current and new immune-modifying strategies will be refined to effectively treat breast cancer.

Keywords: TLR, single nucleotide polymorphism, chemotherapy, radiotherapy, innate immunity

Introduction
Breast cancer, a heterogeneous disease, is a major public health issue for women worldwide. Moreover, it is the leading cause of cancer-related death among women in the USA. In 2012, the American Cancer Society estimated that 226,800 women would receive a breast cancer diagnosis and 39,500 would die from the disease.1 Although there have been improvements in the early detection of breast cancer, a 23% 5-year survival rate plagues women with distant stage disease.2 While breast cancer incidence rates are higher among women of European rather than African descent in the USA, African-American women are more likely to die from this disease. Major breast cancer risk factors include increasing age, overweight/obesity, weight gain after age 18, hormone replacement therapy, alcohol consumption, high breast tissue density, high bone mineral density, exposure to high-dose radiation to the chest, null parity, long menstrual history, having one’s first child after age 30, family history of breast cancer, and inheritance of susceptibilities in high (BRAC1, BRAC2) and low penetrance genes. Recently, studies have suggested that imbalances in inflammatory and immune-associated proteins may also contribute to breast cancer and disease progression.3–6

The immune system consists of both innate and adaptive immune response pathways.7,8 Adaptive immunity is a finely tuned, highly selective response mechanism mediated by antigen-specific T and B lymphocytes in response to infection. In
contrast, the evolutionary conserved innate immune system undergoes activation in response to nonspecific exogenous and endogenous insults that possess pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Of the two inflammatory divisions, innate immune responses serve as the first line of defense.

Several cellular mechanisms, including the toll-like receptor (TLR) pathway, play a crucial role in the innate immune system.\(^9,10\) In particular, TLR signaling pathways promote survival, proliferation and apoptosis, as well as interferon (IFN), cytokine, and chemokine production. However, there is strong evidence showing that infectious agents activate TLRs and promote tumor progression in gastric cancer, Kaposi’s sarcoma, hematological malignancies, and prostate cancer.\(^11–13\) Although pathogenic-induced breast cancer remains inconclusive,\(^14–20\) TLR ligand stimulation can either enhance or inhibit tumor growth, as summarized in Table 1.

Recently, single nucleotide polymorphisms (SNPs) within the TLR signaling pathway have garnered a great deal of attention as candidate cancer detection, prognostication, and clinical management tools. It is speculated that genetic susceptibilities in TLR targets may alter the sensitivity of TLRs to their ligands, which may modify downstream signaling, including release of inflammatory and immune responses that favor tumor development. While it is feasible that TLR sequence variants are related to breast cancer outcomes, this research focus remains understudied.

In this review, we evaluate the TLR signaling pathway in relation to TLR breast cancer risk, disease progression, and tumor behavior. In particular, we highlight the clinical impact of synthetic TLR ligands alone or in combination with other therapeutic regimens on breast tumorigenesis. Lastly, we propose future research avenues to explore the contribution of innate immune mechanisms that support tumor progression and new treatment strategies that target these pathways.

**TLR signaling pathway**

TLRs are essential type I transmembrane glycoproteins that regulate inflammatory responses against harmful pathogens, as shown in Figure 1.\(^21–24\) In humans, at least ten members have been identified and categorized as cell surface or intracellular TLRs based on their substrate specificity. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are cell surface receptors, while TLR3, TLR7, TLR8, and TLR9 are embedded in lysosomal, endosomal, as well as endoplasmic reticulum membranes.\(^15\) Cell surface receptors primarily recognize lipid or protein PAMPs and DAMPs in response to infection and tissue damage, respectively.\(^25\) In contrast, secretory pathway receptors are triggered by DAMPs and nucleic acids, which are presumably released during cell lysis and sequestered in endolysosomes for degradation.\(^26–28\) Accessory molecules for TLR ligand recognition, subcellular localization and/or function have been identified for TLR2, TLR3, TLR4, TLR6, TLR7 and TLR9 (reviewed in Lee et al, 2012).\(^29\)

With the exception of TLR3, which uses the MyD88-independent pathway and TLR4, which uses both the MyD88-dependent and -independent pathways, activated TLRs signal via the MyD88-dependent pathway. Following stimulation, TLRs bind to the sorting adaptor complex toll/interleukin receptor homology domain adaptor protein/myeloid differentiation factor 88 adapter-like protein (TIRAP/MAL), which recruits MyD88 and subsequently activates regulatory serine/threonine interleukin-1Receptor-

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**Table 1 Impact of compounds that affect toll-like receptor TLR2 and TLR4 activity or expression in breast cancer**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ligand type</th>
<th>Biological function</th>
<th>Impact on breast cancer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGB1</td>
<td>Endogenous DAMPs</td>
<td>Critical regulator of cell death and survival</td>
<td>Upregulated in most types of tumors; greater expression in tumors than normal epithelium</td>
<td>Tang et al(^{21}); Lotze and DeMarco(^{24}); Lotze and Tracey(^{25}); Xie et al(^{26})</td>
</tr>
<tr>
<td>PGN</td>
<td>Exogenous PAMPs</td>
<td>Forms cell wall of bacteria</td>
<td>Promoted breast cancer cell invasiveness and adhesiveness in MDA-MB-231 breast cancer cells</td>
<td></td>
</tr>
<tr>
<td>Serum amyloid A</td>
<td>Endogenous DAMPs</td>
<td>Potent chemoattractant</td>
<td>Elevated in breast cancer patients depending on stage</td>
<td>Hiratsuka et al(^{27}); Zhang et al(^{28}); Sfondrini et al(^{29}); Cai et al 2011 (^{10})</td>
</tr>
<tr>
<td>Flagellin</td>
<td>Endogenous PAMPs</td>
<td>Forms flagella in bacterial flagellum</td>
<td>Suppresses cell proliferation and growth in breast cancer cells</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** DAMPs, damage-associated molecular pattern molecules; HMGB1, high-mobility group protein B1; PAMPs, pathogen-associated molecular patterns; PGN, peptidoglycan.
associated kinases (IRAKs). Four IRAKs participate in TLR signaling, namely IRAK1, IRAK2, IRAKM (IRAK3), and IRAK4. IRAK4 is the central regulatory, kinase, signaling its own autophosphorylation as well as phosphorylation of IRAK1, IRAK2, and tumor necrosis factor receptor-associated factor 6 (TRAF6), as depicted in Figure 1.30–32 After IRAK1 dissociates from toll-interacting protein (TOLLIP) it binds and activates phospho-TRAF6, which in turn activates mitogen-activated protein (MAP) kinases and nuclear factor-kappa B (NF-κB). As a result, several transcription factors, including NF-κB and activator protein-1, undergo nuclear translocation, resulting in the secretion of proinflammatory chemokines and cytokines (ie, tumor necrosis factor-α, interleukin [IL]-6, and IL-8).33–36 as well as the induction of interferon-regularatory factors (IRFs, IRF1, IRF3, IRF5, IRF7, and IRF8) and matrix metalloproteinases (MMPs). Besides chemokine and cytokine production, IRAK activation stimulates the Fas-associated protein with death domain (FADD) expression and thereby promotes programmed cell death (apoptosis) via activation of caspase-8 and -10.37,38

The MyD88-independent signaling pathway via activated TLR3 and TLR4 recruits the sorting adaptor toll/interleukin receptor domain-containing adaptor inducing interferon (TRIF) and the signaling adaptor toll/interleukin receptor domain-containing adaptor inducing interferon-related adaptor molecule (TRAM). Association of the TRIF/TRAM complex coupled with ligand-bound TLR3 or TLR4 upregulates IRF3/7 via NF-κB activation but intermediates in the MyD88-independent signaling pathway
have not been well-characterized. The targeted activation/inhibition of either MyD88-dependent or -independent pathways should prove a useful approach to altering the innate immune system's complex autocrine and paracrine induction of growth and immune cell recruitment and tumor cell biology.

The TLR signaling pathway and breast tumorigenesis

Several TLRs, expressed in breast cancer cell lines and/or archival tissue, have been evaluated in relation to tumor behavior. Xie et al observed modest mRNA levels of TLR1, TLR2, TLR3, TLR5, TLR6, and TLR8 but not of TLR4, 7, 9, and 10 in MCF-7 and MDA-MB-231 cells. Moreover, higher levels of TLR2 or its gene product were present in invasive MDA-MB-231 breast cancer cell lines when compared with MCF-7 cells, based on quantitative reverse transcriptase polymerase chain reaction, (RT-PCR) protein analysis, and flow cytometry. In MDA-MBA-231 cells only, TLR2 inactivation positively influenced cell invasion through a variety of mechanisms including: NF-κB activation, which plays a central role in breast cancer progression; induction of phosphorylation of nuclear hormone receptor NR2C2 and 1 kappa B in the TLR2/NF-κB signaling pathway; and secretion of IL-6, VEGF and, MMP-9. TLR3 activation inhibited tumor growth by inducing pro-apoptotic and inhibiting cell proliferation in in vitro and in vivo studies. Unlike Xie et al analysis of all TLRs in MDA-MB-231 cells, Yang et al confirmed the presence of only four TLR mRNAs and proteins (TLR4 > TLR6 > TLR5 > TLR7), in this cell line. Yang et al also demonstrated that TLR4 silencing inhibited MDA-MB-231 cell proliferation and inflammatory IL-6 and IL-8 secretion. Commensurate with Yang’s findings, González-Reyes et al revealed that high expression of TLR4 was associated with disease recurrence, large tumor size, and distant metastatic disease in a case-only study of 74 women. In this same study, TLR3 and TLR9 were also linked to tumor size and tumor stage, respectively. In two independent studies, expression and/or ligand stimulation of TLR9 by CpG oligonucleotides promoted: cell migration in an estrogen-receptor negative BT-20 cell line, cell invasion mediated by MMP-13 in MDA-MB-231 breast cancer cells but not MCF-7 cells, and aggressive tumor behavior. Notably, TLR9 expression in 124 breast cancer tissue specimens collected from women correlated with estrogen-receptor negative/progesterone-receptor negative status, high tumor grade, and NF-κB expression. Conversely, overexpression of TLR9 in fibroblast cells was associated with low propensity toward metastatic breast cancer. The conflicting pro- and anti-tumorigenic properties of TLR9 may be attributed to its role in both cell survival and apoptosis, respectively; therefore, targeting TLR9 requires evaluation and validation in preclinical studies. The role of TLRs in tumor development and inhibition may depend on the cell type, tumor subtype, tumor microenvironment, and the metabolic condition of the cell.

Collectively, these reports suggest that the TLR signaling pathway in breast tumor cells may play a supporting role in the secretion of proinflammatory cytokines/chemokines, aggressive tumor behavior (eg, NF-κB activity), cell proliferation, cell invasion, cell migration, and metastasis. Future inclusion of an adequate representation of breast tumor subtypes, breast cancer cell lines, transgenic animal models, and downstream signaling TLR markers will enhance our understanding of the biological mechanisms that mediate the role of the innate immunity pathway in breast tumorigenesis. Lastly, studies on the silencing of invasive TLRs (eg, TLR2) hold potential for targeted treatment strategies (see the “TLR pathway and breast cancer treatment” section below).

TLR signaling sequence variants and breast cancer risk

Six published reports have examined the relationship between TLR-associated sequence variants and breast cancer. In a pilot study, Etokebe et al did not observe any significant differences in the genotype frequencies of five TLRSNPs detected in TLR3 (T597C, T3350C), TLR3 (C1377T), TLR4 (rs4986790, A1396G, Asp299Gly), and TLR9 (A1359G) between 130 breast cancer cases and 101 controls in a case-control study from Croatia. Contrary to the findings of Etokebe et al, Theodoropoulos et al found that inheritance of the TLR4 rs4986790 Asp299Gly allele was linked to a modest increase in breast cancer risk (odds ratio [OR] = 1.67, 95% confidence interval [CI] = 1.17–2.38) among women from Athens, Greece. However, in the same study, null findings were observed for the TLR4 Thr399Ile SNP.

In the Greek study, the increased susceptibility affiliated with the TLR4 Asp299Gly SNP may be attributed to a reduction in the recognition of TLR4-associated ligands, such as lipopolysaccharide, which is found in the cell wall of Gram-negative bacteria. It is believed that individuals who possess the hyporeactive TLR4 variant allele may have reduced cytokine production, increased susceptibility to acute bacterial
infections, and increased mortality.\(^5\) Alternatively, the low-functioning TLR4 locus may be linked to a compromised inflammatory response that permits damaging persistent subclinical infection.\(^6\)–\(^8\) In any event, genetic alterations attributed to the TLR4 Asp\(^{299}\)Gly SNP may disturb the TLR signaling pathway and enhance proinflammatory networks that favor tumor growth.

The TLR4 Asp\(^{299}\)Gly variant allele is 10–20 times more prevalent among women of African ancestry relative to those of other ancestries.\(^3\) Moreover, this same variant is two to three times more common among African-Americans than among Caucasians.\(^9\) Despite this disparity and the relatively high breast cancer mortality rate among African-Americans and Africans,\(^9\) the influence of the TLR4 Asp\(^{299}\)Gly locus on breast cancer outcomes in these subgroups is currently unknown. Consequently, additional studies are warranted to evaluate the impact of polymorphic TLR4 and other innate immunity markers in relation to breast cancer risk and ultimately disease prognosis among women of African ancestry.

In a secondary analysis of data collected by the National Cancer Institute Cancer Genetic Susceptibility Breast Cancer Genome-Wide Association Study, Kimbro et al analyzed 127 TLR SNPs in relation to breast cancer risk among 1145 postmenopausal women of European ancestry with invasive breast cancer and 1142 controls from the Nurses’ Health Study.\(^30\) Unfortunately, only one marker (TLR6 rs1039559 C/T) was marginally related to a reduction in breast cancer risk. However, this relationship did not remain significant after adjusting for multiple hypothesis testing. In two other large case-control studies, inheritance of the TLR1/TLR6 rs7696175 CC genotype was associated with a 1.6- and 4-fold increase in breast cancer risk among African-American (OR = 4.11; 95% CI = 1.28–13.24) and Chinese women (OR = 1.63; 95% CI = 1.10–2.41), respectively.\(^61\) However, this TLR1/TLR6 locus was not an important indicator of disease risk among European-American women.\(^61\)

An independent study analyzed the relationship between downstream TLR-associated SNPs and breast cancer susceptibility. Among 1536 evaluated SNPs, the additive genetic model of the IRAK3 rs1732877 T\(^{147}\)C SNP was linked to a modest 1.6-fold increase in breast cancer risk (OR = 1.63, 95% CI = 1.14–2.34) among Koreans.\(^63\) Unfortunately, this downstream signaling marker did not remain significant after adjusting for multiple comparison bias.

Taken together, the epidemiologic evidence for the impact of genetic alterations in innate immunity markers relative to breast cancer is not very compelling. However, the lack of strong relationships may be partly attributed to the failure to evaluate higher order gene–gene and gene–environment interactions. For instance, exposure to environmental chemicals may alter innate immunity signaling activities and ultimately modify the relationship between genetic susceptibilities and breast cancer risk.\(^64\) To date, studies have focused primarily on nine SNPs detected in TLR-associated genes (TLR1–4, TLR9, and IRAK3), ignoring hundreds of known and novel TLR downstream signaling markers. Future studies are needed to consider the full array of TLR-related sequence variants and environmental exposures in relation to breast cancer risk within large and racially/ethnically diverse multicenter studies.

**TLR pathway and breast cancer treatment**

Over the past 30 years, a few research efforts have taken advantage of genomic anomalies associated with TLRs to both identify candidate therapeutic targets and design treatment strategies against breast cancer. To date, agonists for TLR3 and 7 have been used to directly inhibit tumor growth in preclinical studies or to treat breast cancer in humans.\(^49\) In addition, TLR3 and TLR4 expression or functional TLR4 sequence variants appear to influence tumor sensitivity to chemotherapy,\(^43,71\) albeit unequivocally.\(^72\) Lastly, stimulation of TLR9 is the focus of many ongoing randomized clinical trials to treat breast cancer.

Synthetic double-stranded RNAs (dsRNAs), including polyinosinic:polycytidylic and polyadenylic:polyuridylic acid (Poly A:U) have been used in recent breast cancer preclinical and clinical trial studies.\(^65\) The use of dsRNA Poly A:U, which targets TLR3 expressed on breast tumor cells, has resulted in favorable breast cancer outcomes (ie, an increase in overall survival and a decrease in metastasis) in two randomized clinical trials with 14 or 111 months of follow-up.\(^66,67\) Similarly, in a randomized trial of dsRNA treatment versus chemotherapy, Salaun et al demonstrated a decrease in metastatic relapse among TLR3 positive but not TLR3 negative breast cancer patients in the Poly A:U arm.\(^4\) Among patients with a TLR3 positive tumor, women in the Poly A:U arm were three times more likely to remain metastasis free 15 years post-treatment than those treated with chemotherapy (56% [95% CI = 37–71] vs 20% [95% CI = 8–37]). The anti-tumorigenic properties of Poly A:U were supported with results using a breast adenocarcinoma cell line and an immune-compromised mouse model. Following exposure to Poly A:U, the HCC1806 cells underwent TLR3-dependent cell death. Further, subcutaneous growth of the HCC1806...
Cell lines was compromised by intravenous injection of Poly A:U within immune-compromised non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mice relative to controls. Although the exact mechanisms are unclear, dsRNAs may elicit their antitumor properties against breast cancer by: reducing cell proliferation; inducing TLR3 (and TRIF)-dependent cell death, independent of dsRNA-dependent protein kinase; and promoting IRAK4 induction of IFN-β, NF-κB, and – to a lesser extent – caspase-3 and -8 activation. Although there are no specific data for breast cancer, we cannot exclude the possibility that dsRNAs may potentiate the capacity of TLR3 to trigger a chemoattractant response (secretion of chemokines, macrophages, neutrophils, and lymphocytes) to eradicate the tumor or alter tumor vascularization. In any case, participation of innate and adaptive immunity is critical to the success of chemotherapy against breast cancer.

Although attention has been given to targeting the TLR signaling pathway for breast cancer treatment, it is important to note that this pathway influences sensitivity to some, but not all, chemotherapeutic strategies. In a breast cancer murine model, Stimulation of dendritic cell TLR4 in combination with systemic chemotherapy or local radiotherapy was shown to reduce tumor growth and prolong survival of tumor-bearing mice. Apetoh et al demonstrated that breast cancer patients who carried a TLR4 loss of function SNP (TLR4<sup>Asp/Gly</sup> + Gly/Gly) were more likely to experience metastatic recurrence after chemotherapy and local radiation relative to carriers of the referent genotype. In contrast, Szkandera et al did not observe a significant relationship between two commonly reported TLR4 SNPs (Asp<sup>Gly</sup> and Thr<sup>199Ile</sup>) and pathological response to neo-adjuvant anthracycline-based chemotherapy among breast cancer patients. However, this null finding may be due to the small sample size (70 Caucasian female patients) as well as failure to consider other TLR-associated sequence variants.

In a subcutaneous setting, the administration of TLR5 ligand (Salmonella typhimurium flagellin) in the D2F2 mouse mammary tumor cell line and its antigenic sub-line with stably transfected ErbB-2 (DF2/E2) resulted in varied breast outcomes depending on the dosing regimen. Tumor growth was inhibited only in the D2F2 cell line when flagellin was administered 8–10 days after tumor inoculation. However, accelerated tumor growth was observed for both cell lines when the ligand was administered at the time of inoculation. These findings suggest that the impact of TLR5 in relation to breast tumorigenesis is influenced by interactions among the tumor, the tumor microenvironment, and the host immune system. In addition, a combination of TLR5 and TLR9 agonists (i.e., CpG oligonucleotides) [CpG ODN] administered 8–10 days after inoculation, inhibited tumor growth in D2F2/E2 cell lines. Additional studies are warranted on the stimulation of multiple TLRs and their joint effects on abrogating tumor growth.

TLR7 recognizes single-stranded RNA and triggers innate immune signaling in response to viral infections. TLR7 is also activated by several low molecular weight compounds, including imiquimod, which has anti-tumorigenic properties: induction of cytokines (IFN-γ, IL-12), activation of tumor antigen-specific cytotoxic T-lymphocytes, and activation of myeloid dendritic cells with cytotoxic activity. A topical cream formulation of imiquimod has been approved by the US Food and Drug Administration for the successful treatment of superficial basal cell carcinoma, actinic keratosis, and genital warts. Within ongoing cancer trials, topical application of imiquimod targets several cancers including breast cancer. For instance, the New York University School of Medicine is currently running a Phase II/III clinical trial with imiquimod for breast cancer patients with chest wall recurrence and skin metastasis. In addition, the Fred Hutchinson Cancer Research Center recently completed a Phase III trial of imiquimod in conjunction with a paclitaxel albumin-stabilized nanoparticle formulation.

Despite the conflicting roles of TLR9 in tumorigenesis, TLR9 agonists (eg, CpG ODN) have been studied as antitumor drugs either alone or in concert with other therapeutic strategies in preclinical and clinical studies. Stimulation of TLR9 in CpG DNA triggers tumor invasion and migration in breast cancers using in vitro assays. Currently, several clinical trials are capitalizing on the antitumorigenic properties of the TLR9 agonist CpG 7909 alone or in conjunction with trastuzumab. It is speculated that TLR9 ligands may stimulate anti-tumorigenic activity by inducing apoptotic signals or by interfering with blood vessel formation and tumor growth; however, the exact mechanism for breast cancer remains unclear.

The growing understanding that TLR-associated targets are involved in breast tumorigenesis offers some justification using larger pharmacogenetic studies. However, such observational studies need to consider the full cadre of possible targets involved in the TLR signaling pathway (TLRs, adaptor/accessory proteins, IRAKs, MMPs, chemokines, cytokines, and caspases). Collectively, continuing efforts targeting the TLR signaling pathway may lead to more effective, noninvasive strategies that will improve breast cancer survival rates.
Conclusion
Breast cancer is a heterogeneous disease whose incidence and progression may vary according to genetic status and function of the tumor innate immunity system. Basic research on key proteins and genetic variants in the TLR signaling pathway will likely offer insights into the processes that promote and/or prevent breast tumorigenesis. Therapeutic targeting of the TLR signaling pathway may be used in conjunction with new or existing chemotherapy, radiation, or immunotherapy approaches to treat breast cancer. Future studies targeting multiple TLRs may lead to more effective therapeutic targets for drug development and treatment of breast cancer. Such effort may ultimately improve breast cancer survival rates among all women.

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Disclosure
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