The Biology of Toll-Like Receptor 9 and Its Role in Cancer

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ABSTRACT: Toll-like receptor 9 (TLR9) plays a fundamental role in innate immune responses through pathogen-associated and danger-associated molecular pattern recognition. Ligand recognition by TLR9 results in activation of several signaling pathways, including those involving nuclear factor-kappa B, mitogen-activated protein kinases, and interferon-regulatory factors, which promote secretion of proinflammatory cytokines and type I interferons. TLR9 is expressed by immune-mediated cells and in clinical specimens and cell lines of various human cancers. TLR9 appears to act as a double-edged sword in cancer, with some studies indicating that it is associated with increased malignancy and others indicating that it contributes to immune response against cancer. At present, the mechanisms underlying the role of TLR9 in cancer pathophysiology are not completely clear, although various TLR9 agonists and antagonists are being tested in *in vitro* and *in vivo* cancer models as well as clinical trials. This review summarizes the current state of knowledge regarding TLR9 features, isoforms, structure, ligands, and signaling, and discusses the roles of TLR9 in cancer pathogenesis. Recent efforts to utilize TLR9 agonists and antagonists as potential anticancer immunotherapy agents are also highlighted.

KEY WORDS: TLR9, signaling, adaptive immunity, cancer, SNPs

I. INTRODUCTION

Innate immunity is a first-line defense that protects the body from harmful pathogens. For some time, the molecular basis of the innate immune system and mechanisms underlying inflammatory factor production were unclear. However, after Toll-like receptors (TLRs) were discovered to play crucial roles in immune system response to pathogens, studies of TLRs have rapidly advanced our understanding of their biological and pathophysiological roles. The discovery of TLRs in the late 1990s was an important event in the field of immunology and was recognized by a Nobel prize awarded to Jules Hoffmann, Bruce Beutler, and Ralph Steinman in 2011.[1]

Pattern recognition receptors are expressed by cells of the innate immunity system. TLRs are believed to be the most ancient subgroup of pattern recognition receptors because they represent the most extensive spectrum of molecule recognition.[2] Innate immunity begins with the recognition of pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) by pattern recognition receptor subgroups, including TLRs.[2]

The Toll protein was first discovered in the adult fruit fly.[3] So far, 13 functional TLRs have been identified, 10 of which are expressed by humans (TLR1–TLR10) and 13 of which are expressed in mice (TLR1–TLR13).[2] TLRs can be differentiated by their ligand specificity, activation of particular signaling pathways, and subcellular localization.[4] Some TLRs (TLR1, TLR2, TLR4–TLR6, TLR10) are located on the outer membrane surface of immune cells and recognize extracellular pathogens, whereas other TLRs (TLR3, TLR7–TLR9, TLR11, TLR13) are expressed on the membrane surfaces of intracellular vesicles and recognize intracellular pathogens.[5,6]

Chronic inflammation and cell death are associated with tumorigenesis, and increased PAMP and DAMP production are seen in various cancers.[7] Cancer cells also acquire features of immune cells, which allow them to regulate the immune response to benefit their own growth and survival. Additionally, the nuclear factor-kappa B (NF-κB) signaling pathway activation that occurs in most tumors stimulates the production of chemokines, cytokines, antiapoptotic molecules, growth factors, and collagenases, which in turn promote tumor initiation, progression, distance metastasis, and chemoresistance.[8]

Interestingly, activation of TLRs can result in both protumor and antitumor responses. TLRs play important roles in cancer cell invasion, immune evasion, survival, proliferation, and distant metastasis, but they also suppress tumor growth and proliferation and stimulate cancer cell apoptosis.[9]

TLR9, also called CD289, is mainly activated by unmethylated cytidine-phosphate-guanosine (CpG) nucleotides. TLR9 is expressed by immune cells, including B cells, dendritic cells, monocytes/macrophages, natural killer cells, T cells, and other antigen-presenting cells.[10] CpG/TLR9 interactions in immune-mediated cells lead to stimulation of the innate immune system via activation of signaling pathways and secretion of proinflammatory cytokines such as type I interferons (IFNs).[7] TLR9 is also expressed by various types of cancer cells and plays important roles in cancer pathogenesis. Like other TLRs, TLR9 has bidirectional effects on the immune system, promoting both tumor progression and regression. This review discusses the accumulating evidence pertaining to TLR9 biology and its role in cancer.

II. TLR9 STRUCTURE

TLR9 is encoded by the *TLR9* gene, which maps to human chromosome 3p21.3. This gene has two exons, of which the second is the major coding area. It is expressed in two spliced forms: monoexonic and biexonic.[11] The TLR9 protein comprises 1,032 amino acids and is 150 kDa in length.

The TLR9 structure has three major components: a leucine-rich repeat (LRR), a transmembrane domain, and an intracellular Toll/interleukin (IL)-1 receptor (TIR) domain.[6] LLR is involved in molecule recognition, and TIR interacts with adaptor and signaling molecules. The TLR9 ectodomain forms a horseshoe-shaped solenoid assembled from 26 LRRs, with each LRR containing around 20–25 amino acids. The ends of the TLR9 ectodomain are capped by a cysteine-rich C-terminus (at the LRR end) and N-terminus (at the TIR end).[12] In TLR9/ligand interactions, TLR9 is cleaved proteolytically at its ectodomain, resulting in TLR9 dimerization and activation.[13] Both terminal regions of TLR9, but mainly the C-terminal region, respond to nucleic acid. CpG DNA interacts with LLR1-10 of the N-terminal region and LRR20-22 of the C-terminal region.[14] TLR9 contains two DNA binding sites—CpG and the 5′ end—for which cooperative binding is required for TLR9 dimerization and activation.[15] The dimerization of TLR9 is also mediated by cleavage of the Z-loop in the middle of the TLR9 ectodomain (between LRRs 14 and 15).[16]

The sensing of ligands by TLR9 is regulated by two mechanisms: (1) trafficking of TLR9 from the endoplasmic reticulum to endosomes and lysosomes with the presence of Unc93 homolog B1 (Unc93B1), a multiple transmembrane protein; and (2) the cleavage of TLR9 in endolysosomes by endopeptidase.[13]

III. TLR9 ISOFORMS

A study of human gene expression reveals that TLR9 has five isoforms—TLR9-A, TLR9-B, TLR9-C, TLR9-D, and TLR9-E[17]—produced by alternative splicing of the transcript. The protein sequences of these isoforms suggest that they have different subcellular localizations: TLR9-A, TLR9-C, TLR9-D, and TLR9-E may localize in the endoplasmic reticulum, whereas TLR9-B may localize in mitochondria. Furthermore, these isoforms have different amino acid sequences and show different expression patterns among immune cell types and across developmental stages.[17]

IV. TLR9 LIGANDS

PAMPs are exogenous microbial pattern ligands recognized by TLRs. Examples of PAMPs include lipopolysaccharide from gram-negative bacteria; lipoteichoic acid and peptidoglycan from gram-positive bacteria; flagellin from bacterial flagella; lipoarabinomannan, lipoglycans, and lipomannans from mycobacterium; zymosan from yeast; and double-stranded and single-stranded RNA from viruses.[4] TLR9 detects unmethylated 2′-deoxyribo CpG DNA originally sourced from bacteria, viruses, and protozoa.[18]

DAMPs are endogenous biomolecules that are also considered to be TLR ligands. DAMPs are secreted as a result of injury and cell death. Examples of DAMPs include heat shock proteins,

high-mobility group box protein 1 (HMGB1), mitochondrial DNA and other organelles, extracellular matrix components, and plasma membrane constituents.[4]

TLR9 recognizes single-stranded DNA consisting of 21 or more nucleotides with a TCG or TCC sequence at the 5′ end plus the CpG motif.[15] TLR9 is also weakly activated by methylated single-stranded or double-stranded DNA.[16] Chromatin IgG complex secreted from the nucleus acts as a ligand stimulating TLR9 activation.[19] Furthermore, TLR9 is activated by the CpG content molecule known as mitochondrial (mt)DNA, a DAMP member. TLR9 and mtDNA interactions induce a signaling cascade that results in an immune response.[20] Today, synthetic nonmethylated CpG-containing oligodeoxyribonucleotide (ODN) motifs with various sequence lengths and secondary structures can catalyze TLR9 activation via natural substrates.[21]

V. TLR9 SIGNALING

Interactions of TLRs with ligands, adaptors, and other receptors and coreceptors activate signaling pathways that regulate the gene transcription of inflammatory factors that mediate innate and adaptive immunity, such as cytokines and chemokines, antimicrobial peptides, and reactive oxygen species.[22] CD14 is a membrane-associated protein that works as a dimeric coreceptor for TLR9.[23] Like other TLRs, it also contains LRRs and has shared ligand-binding properties. CD14 both stimulates the internalization of DNA and promotes DNA delivery to endosomes and the ability of TLR9 to be triggered by ligands.[23]

Seven adaptors—myeloid differentiation 88 (MyD88), MyD88 adaptor-like (MAL), TIR domain-containing adaptor-inducing interferon-β (TRIF), TRIF-related adaptor molecule, sterile-α and armadillo motif-containing protein, B cell adaptor for PI3-kinase, and src kinase-interacting membrane protein (SCIMP)—are involved in the downstream signaling initiation of TLRs.[24] All of these adaptors contain homotypic TIR that links directly to TLRs except for SCIMP, which binds to TLRs by a typical TIR/non-TIR interaction.[24]

MyD88, MAL, and SCIMP are the three adaptors that regulate signaling of TLR9.[24] Upon TLR9 recognition, a myddosome complex is formed composed of activated MyD88, IL-1 receptor-associated kinase-1 (IRAK1), and IRAK4, and control subsequent recruitment of tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6). TRAF6, in the presence of ubiquitin-conjugating enzymes UBC13 and UEV1A, activates K63-linked polyubiquitination of TRAF6 itself and the TAK1 complex associated with its TAB1, TAB2, and TAB3 subunits. Activation of TAK1 leads to activation of mitogen-activated protein kinase (MAPK), activator protein 1 (AP-1), and NF-κB signaling pathways.[22,23] NF-κB and AP-1 transcription factors then upregulate transcription of cytokine genes (e.g., IL-6, IL-12, and TNF) and costimulatory molecules (e.g., CD80 and CD86).[25] Furthermore, TLR9 activation in dendritic cells (DCs) is responsible for production of type I IFNs. Induction of type I IFNs is regulated, in turn, by the interaction between MyD88 and interferon regulatory factor-7 (IRF7) signaling involving IRF7, IRAK 4, IRAK1, and TRAF6.[26,27]

The MAL adaptor also precipitates TLR9 signaling pathways, as TLR9 signaling is inhibited in the absence of MAL in herpes simplex virus 1 (HSV-1) infection.[28] Although the signaling downstream of the TLR9/MAL interaction has not been elucidated, MAL participates in the activation of NF-κB, IRF5, and AP-1, ultimately culminating in the production of proinflammatory cytokines through TLR2 and TLR4.[29]

Besides MyD88 and MAL, SCIMP is considered an adaptor involved in TLR9 signaling.[24] This adaptor brings Lyn to TLRs during cellular activation, leading to activation of downstream signaling and promotion of IL-6 and IL-12p40 production in macrophages[30] (Fig. 1).

The involvement of TLR9 in the production of type I IFNs is regulated by stimulation of the phosphoinositide 3-kinase (PI3K) signaling pathway.[28] Additionally, the interaction between TLR9 and the MyD88 adaptor is blocked and IFN-α/β production is impaired after pharmacological inhibition of the PI3K-mammalian target of rapamycin-p70 ribosomal S6 protein kinase.[28]

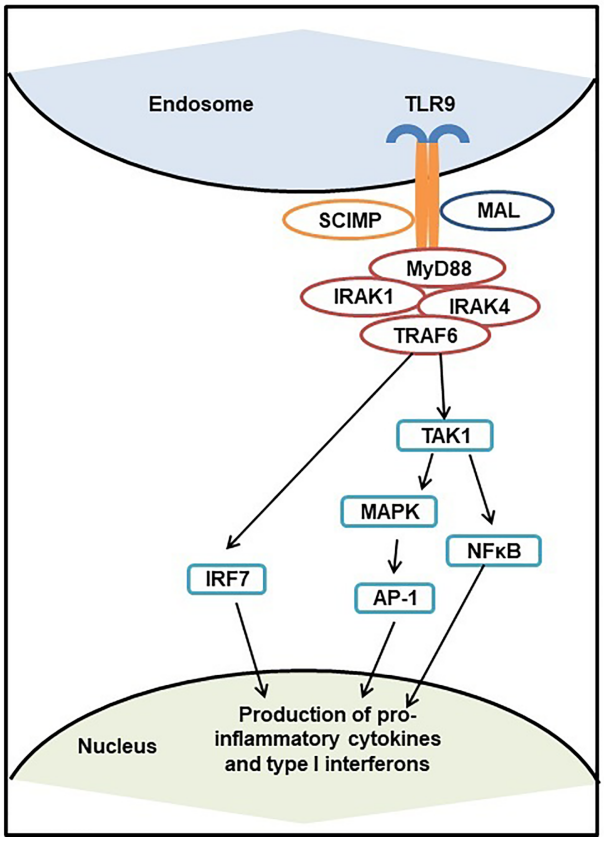

FIG. 1: Schematic of TLR9 signaling pathway. TLR9 interacts with intracellular adaptors MyD88, MAL, and SCIMP. At TLR9/ligand binding, MyD88 binds with intracellular molecules IRK1, IRK4, and TRAF6, which are activated sequentially. TRAF6 then activates the TAK1 complex, which undergoes autophosphorylation, and activates downstream signaling pathways involving MAPK and NF-κB, increasing the expression of pro-proinflammatory factors. IRF7 activation is also mediated by MyD88/TRAF6, which results in production of type I interferons.

VI. TLR9 IN CANCER

A. Liver Cancer

TLRs are normally expressed by liver cells and play important functions in the healthy liver, although expression of TLRs, including TLR9, is weaker in the liver than in other organs.[31] In the liver, TLR9 is expressed by hepatocytes, stellate cells, Kupffer cells, DCs, and sinusoidal endothelial cells.[32] However, TLR9 is highly overexpressed in hepatocellular carcinoma (HCC) cells.[33] During chronic liver diseases, the gut continuously secretes microbial DNA recognized by TLR9 in hepatic tissue, as the gut and liver are anatomically and functionally associated. Also, the death of hepatocytes in the injured liver leads to the release of denatured DNA or mtDNA, which are detected by TLR9.[34] Recognition, activation, and overexpression of TLR9 are highly associated with hepatic inflammation, fibrosis, and HCC progression.[32]

TLR9 positively contributes to the development of liver cancer. TLR9 acts as a tumor activator in HCC by activating its signaling, leading to enhanced cancer cell proliferation and survival.[35,36] In hypoxia-induced HCC, TLR9 is activated by HMGB1, which facilitates tumor growth.[32] HMGB1 expression is elevated in the tumors and circulating blood of HCC patients.[37] HMGB1/TLR9 interactions induce mitochondrial biogenesis in hypoxic HCC cells as well as HCC cell survival and proliferation.[38] Furthermore, liver cancer is eliminated by ablating the TLR9 gene and its downstream signaling molecule MyD88.[32] Additionally, the proliferation and growth of tumor cells is blocked by TLR9 inhibition via IRS-954 or chloroquine.[39] In an HCC cell line, TLR9 activation promotes tumorigenesis by stimulating antiapoptotic molecules (e.g., X-linked inhibitor of apoptosis protein [cFLIP], X-linked inhibitor of apoptosis protein [XIAP], B cell lymphoma-extra large [Bcl-xL], survivin) and modulating oncogenic gene expression.[36] mtDNA is highly released in HCC, and its interaction with TLR9 results in NF-κB activation and tumor-associated macrophage (TAM) recruitment and polarization. TAM cell infiltration is closely related to solid tumor development.[40] The administration of DNase I to deplete mtDNA or blockade of TLR9 by a TLR9 antagonist reduces TAM recruitment and polarization and thereby slows HCC progression.[40]

However, some studies show that TLR9 expression plays an important role in tumor suppression. For instance, transfection with the TLR9 agonist ODN M362 exerts proapoptotic effects in a human HCC cell line and inhibits their proliferation by 50%.[41] Clinical and preclinical studies show that a TLR9 antagonist combined with

another therapeutic agent exerts potent antitumor activity. For example, cotreatment of the TLR9 agonist CpG ODN with poly(I:C) transfection has a weaker proapoptotic effect in HCC than transfection with poly(I:C) alone. This agonist also assists in reducing the expression of poly(I:C)-related receptors, apoptotic factors, and proinflammatory cytokines.[42] In addition, stimulating the activity of TLR9 improved the antitumor effects of radiofrequency ablation in a VX2 tumor animal model. Furthermore, application of CpG B oligonucleotide, a TLR9 agonist, in radiofrequency ablation-treated rats induced an antitumor T cell response and cytotoxicity, inhibited tumor spread, and increased survival rate.[43]

The association of TLR9 polymorphism with liver cancer is not well studied. However, one study linked *TLR9* 1486C/T (rs187084) polymorphism to a lower risk of HCC recurrence after liver transplantation.[44]

B. Brain Cancer

TLR9 is highly expressed in brain cancer,[45,46] and its increased expression is correlated with poor survival of patients with glioblastoma multiforme, a type of brain tumor.[45] TLR9 expression is positively associated with the grade of glioma malignancy, and the TLR9 agonist CpG ODN promotes glioma invasion[45] and the invasion of astrocytoma and glioblastoma cells.[47] The role of TLR9 in cell invasion is confirmed by the observation that cell invasion is reduced after silencing TLR9 in brain cancer cells with stimulated gene expression of tissue inhibitor of matrix metalloproteinase (MMP)3, MMP2, MMP9, and MMP13.[46] In addition, TLR9 is extensively expressed by glioma stem-like cells, which play an important role in glioma growth. The silencing of TLR9 in these cells leads to reduced glioma stem-like cell development, whereas TLR9 overexpression stimulates the activity of janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3). Thereby, it promotes cell growth.[48] Also, TLR9 is associated with a low survival rate for glioma patients, in whom its protein is expressed by glioma cells and elevates MMP-2 and MMP-9 expression.[49]

In contrast, several studies have shown positive effects of TLR9 in brain cancer. CpG ODN stimulates apoptosis of brain cancer cells and inhibits tumor growth and rechallenge. Thus, it may bestow long-term antitumor effects against gliomas in *in vitro* and *in vivo* models.[50] Administration of CpG ODN107 in human glioma cells promotes radiotherapy effects against glioma cancer and increases levels of TLR9, inducible nitric oxide synthase, nitric oxide, and NF-κB activation.[51] In addition, the combination of radiotherapy and CpG ODN107 stimulates autophagic glioma cell death mediated by the TLR9-extracellular signal-regulated kinase (ERK)-mammalian target of rapamycin (mTOR) signaling pathway.[52]

C. Breast Cancer

TLR9 expression is detected in breast milk cells and epithelial cells of the mammary gland[53] and is also expressed by epithelium cancer cells in breast tissue, where its expression is positively correlated with breast cancer development.[53] In fact, TLR9 is the most upregulated TLR in breast tumors, with its overexpression detected at the very early stage of human breast carcinogenesis.[54] It was shown that TLR9 contributes to breast cancer metastasis and invasion.[53] The role of TLR9 in breast cancer invasion is regulated by its ability to stimulate the activity of MMP-13.[47] In one study, sex steroid hormones and estrogen receptor-α were found to mediate the expression of TLR9 in human breast cancer cells and its role in cancer cell invasion, with TLR9 A and B isoforms identified in clinical breast cancer specimens.[55] Necrosis, apoptosis, and active cellular secretion are three processes that regulate the release of DNA molecules from breast cancer cells, and the interaction of these free DNAs with TLR9 may induce the proliferation of breast cancer cells via the TLR9-NF-κB-cyclin D1 pathway activation.[56] DNA molecules released from dead cells after chemotherapy treatment regulate the activity of TLR9 in cancer cells, which diminishes the effectiveness of chemotherapy.[57] Also, two 22-nucleotide DNA fragments sourced from telomeres, a 9-mer hairpin and a G-quadruplex DNA, were shown to act as TLR9 ligands and contribute to cancer cell invasiveness

in an *in vitro* model.[58] Furthermore, low TLR9 expression increases the effects of bisphosphonates, a chemical used to limit breast cancer spreading, as shown in *in vivo* and *in vitro* models.[59]

On the other hand, TLR9 expression in triple negative breast cancer is associated with longer survival of breast cancer patients.[60] The antiangiogenic effect of trastuzumab is enhanced after coadministration of immune modulatory oligonucleotide (IMO), aTLR9 agonist, in trastuzumab-resistant breast cancers associated with suppressed endothelial human epidermal growth factor receptor-related signaling.[61] Additionally, in the human breast cancer cell line T47D, incubation of 17β-estradiol, which inhibits estrogen receptor-α activity, along with the TLR9 agonist CpG ODN, reduces cell proliferation through NF-κB activation.[62]

Genetic variants of TLR9 have been investigated in breast cancer patients. Studies show that a *TLR9* single nucleotide polymorphism (SNP), rs352140, is associated with breast cancer risk.[63] This mutation results in a change in TLR9 protein function and stability but not its structure.[63,64]

D. Cervical Cancer

Cervical cancer is a major gynecological malignancy largely due to human papilloma virus (HPV), which is a DNA virus that infects the uterine cervix. After HPV infection, the host immune system can usually respond to and clear the virus, although a few HPV types can persist and cause cancer.[65] Compared with patients who cleared a particular HPV genotype, TLR9 expression is higher in patients who are persistently infected with that HPV genotype.[66] TLR9 is highly expressed in cervical cancer tissue compared with normal cervical epithelium and plays a role in the progression and transformation of cervical squamous cells.[67,68] Furthermore, TLR9 expression in peripheral blood mononuclear cells and serum is higher in cervical cancer patients than in control donors. Thus, TLR9 can be used as a diagnostic marker for cervical cancer as its level is altered in the periphery.[69]

However, TLR9 can also have a beneficial impact in cervical cancer, reducing the possibility of HPV16 escaping the host immune response. TLR9 downregulation mediated by high-risk HPV16 E6 and E7 oncoproteins is correlated with an abolished innate immune response.[70]

Cervical cancer patients show genetic variations in TLR9. *TLR9* rs187084 polymorphism is correlated with an increased risk of HPV infection and cervical cancer in an Indian population.[71] *TLR9* 1486T/C (rs187084) and G2848A (rs352140) polymorphisms are associated with an overall higher risk of cervical cancer in Caucasian patients and decreased risk of cervical cancer in mixed-ethnicity patients.[72] *TLR9* C296T/Pro99Leu (rs5743844) is not involved in cervical cancer.[73]

E. Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a lymphoid malignancy in which mature B lymphocytes expand and progressively accumulate in bone marrow lymphoid tissue and peripheral blood.[74] TLR9 is expressed by normal human B cells, but its expression increases in CLL B cells.[75] However, another study shows that TLR9 gene expression is lower in CLL patients than in control individuals.[76] CpG ODN/TLR9 interaction is associated with greater NF-κB activity in CLL, resulting in CLL cell survival.[77] TLR9 stimulation by CpG or CpG/CD40L in CLL cells increases expression of enhancer of zeste homolog 2, which plays a role in CLL aggressiveness and cell survival. Stimulation of TLR9, and hence zeste homolog 2, markedly increases levels of antiapoptotic molecules, including Bcl-xL and myeloid cell leukemia 1 (Mcl-1), and cell viability, and reduces levels of proapoptotic cleaved caspase-3 and poly-ADP ribose polymerase.[78] CLL B cell growth is promoted by the TLR9 agonist CpG ODN 2006, which increases the mRNA/protein expression of IL-15 receptors (IL-15Rα and CD122) and activates PI3K/Akt production, which stimulates activation of the JAK/STAT pathway and in turn provokes the apoptosis pathway in CLL B cells.[75] TLR9 stimulation by CpG ODN 2006 improves the protective effect of fludarabine, a therapeutic agent that promotes cell apoptosis in CLL cells by upregulating lymphotoxin-α expression.[79]

F. Lymphoma

Lymphoma is a blood cancer that affects lymphocytes and lymphoid tissue. TLR9 is expressed in several types of lymphoma, including peripheral T cell lymphoma and B cell lymphomas (follicular lymphoma and diffuse large B cell lymphoma [DLBCL]).[80] TLR9 expression is increased in lymphoma patients and is negatively involved in lymphoma development. Elevated TLR9 expression is observed in tissues obtained from angioimmunoblastic T cell lymphoma patients compared with normal T cell tissue, and higher TLR9 expression is associated with a reduced survival rate.[81] In DLBCL patients, TLR9 and its signaling pathways, NF-κB, STAT3, and p38, are activated and associated with increased migration and proliferation of cancer cells.[82] This activation of TLR9 signaling is stimulated by neutrophil extracellular traps, which are complexes of chromatin DNA and proteins released from neutrophils to trap microorganisms. Additionally, TLR9 inhibition reduces lymph node metastasis and tumor growth in DLBCL.[82]

On the other hand, TLR9 and its ligands are positively recruited in lymphoma suppression. B cell lymphoma regression in human patients is induced after *in situ* vaccination with the TLR9 agonist PF-3512676 in patients undergoing radiotherapy.[83] Similarly, the TLR9 agonist SD-101 enhances indolent lymphoma patients' response to radiation, with decreases in the number of $CD8^+$ T cells, $CD4^+$ regulatory T cells (Tregs), and granzyme B^+ $CD8^+$ T cells in the tumor microenvironment.[84] CpG STAT3 dODN, composed of the TLR9 agonist CpG 7909 and a STAT3 inhibitor, suppresses the growth of human OCI-Ly3 non-Hodgkin's lymphoma in immunodeficient mice. Administration of this compound prolongs survival and protects mice from tumor recurrence.[85] The effects of ibrutinib, an inhibitor of Bruton's tyrosine kinase, on the antitumor immune response in mouse lymphoma is promoted by intratumoral injection of CpG ODN.[86] Intratumoral administration of IMO-2125 in mice injected with the A20 B cell lymphoma line exerts antitumor activity by increasing $CD3^+$ T lymphocytes and upregulating selected immune checkpoint genes such as indoleamine 2,3-dioxygenase-1 (IDO-1), cytotoxic T lymphocyte–associated protein-4 (CTLA-4), and programmed cell death protein ligand-1(PD-L1).[87] Induced cell apoptosis is seen in Burkitt's lymphoma cells after treatment with ODN CpG 2006.[88]

The *TLR9* gene polymorphisms −1237C and 2848A are associated with risk of Hodgkin's lymphoma development.[89] In addition, the *TLR9* 1237C (rs5743836) polymorphism is correlated with an overall increased risk of non-Hodgkin's lymphoma in Portuguese and Italian populations. TLR9 transcriptional activity is stimulated in mononuclear cells from patients harboring the *TLR9* 1237C polymorphism, further supporting the association of this polymorphism with a greater risk of non-Hodgkin's lymphoma.[90]

G. Colorectal Cancer

TLR9 expression is detected in colorectal cancer and is reduced in hyperplastic and villous polyps in colorectal cancer tissue compared with control tissue.[91] TLR9 plays a key role in colon tumor recurrence after radiotherapy. TLR9 inhibition in a mouse model of CT26 colon cancer results in delayed tumor regrowth after administration of high-dose tumor irradiation. The role of TLR9 in tumor recurrence is mediated by activation of MyD88/NF-κB, which increases IL-6 production. Also, TLR9 stimulates Jak/STAT3 signaling, which regulates tumor-promoting inflammation and revascularization.[92]

Several lines of evidence suggest that TLR9 exerts antitumor effects against colorectal cancer. TLR9 expression serves a protective role against malignant transformation in colorectal cancer.[91] Coadministration of the TLR9 agonist IMO and cetuximab reduces the survival of colorectal cancer cells and completely suppresses MAPK phosphorylation in mice and the human colorectal cancer LS174T cell line.[93] IMO administration enhances the effect of cetuximab on antibody-dependent cell-mediated cytotoxicity.[94] Also, IMO plus bevacizumab, an antivascular endothelial growth factor (VEGF) antibody, has antitumor potential in mice xenografted with GEO, LS174T, and GEO-CR. This treatment combination inhibits cancer cell proliferation and angiogenesis, AMP-activated protein kinase (AMPK)

and Akt activation, and VEGF expression.[94] TLR9 agonists CpG ODN and IMO reduce the proliferation and survival of colon cancer cells, increase their apoptosis, and enhance the effects of chemotherapy and radiation.[84] In colitis-associated colon cancer, TLR9 activation via an interaction with X-DNA (X_S-DNA and X_L-DNA) is associated with an enhanced immune response involving production of cytokines and costimulatory molecules by DCs.[95] Also, TLR9 activates MAPK and NF-κB via X-DNA and thereby enhances the effects of doxorubicin, an anticancer therapy.[95] Cooperation between CpG 1826 and α-galactosylceramide-loaded tumor cells (tumor-Gall) enhances protective immune responses and antitumor effects in a mouse colon cancer model, which helps reduce tumor growth and prolong mouse survival. Administration of CpG1826 plus tumor-Gal activates the production of IFN-γ by invariant natural killer (NK) T cells and secretion of IL-12 by DCs.[96] Following chemotherapy, treatment with the TLR9 agonist MGN 1703 improves progression-free survival and activates NK T cells in patients with metastatic colorectal carcinoma.[97]

TLR9 gene polymorphisms are associated with the risk of colorectal cancer. *TLR9* T1237C and T1486C polymorphisms detected in colorectal patients are associated with metastatic disease and shorter survival.[98] In addition, the *TLR9* rs187084 SNP is markedly associated with colon cancer risk selectively in women, and *TLR9* rs352139 and rs352144 SNPs are associated with colorectal cancer progression and localization in Saudi Arabian patients.[99]

H. Gastric Cancer

TLR9 is overexpressed in specimens collected from gastric cancer patients.[100] DNA in *H. pylori*, an important risk factor for development of gastric cancer, encodes a type IV secretion system that plays a fundamental role in *H. pylori* DNA translocation as well as TLR9 overexpression and activation.[101] *H. pylori* infection results in increased TLR9 expression in gastric cancer patients compared with healthy individuals,[102,103] and is associated with increased expression of IL-8, IL-10, and TNF-α.[102] Inflammatory cytokine and chemokine production is mediated by *H. pylori* DNA recognition by TLRs, including TLR9.[104] In gastric cancer, *H. pylori* increases expression of cyclooxygenase-2 (COX-2), which promotes cancer cell invasion and angiogenesis via TLR9.[105] *H. pylori* DNA and TLR9 interaction stimulate p38 MAPK activation and downstream transcription factors, leading to Cre and AP-1 activation on the promoter of COX-2.[105] Mutant TLR9 inhibits *H. pylori*-induced COX-2 expression and promoter activity.[106] Working through TLR9, *H. pylori* activates phosphatidylinositol-specific phospholipase C gamma (PI-PLCγ), protein kinase **C**-alpha (PKCα), c-Src, IκB kinase (IKK)α/β, and NF-κB–inducing kinase (NIK) pathways, which in turn regulate NF-κB activation and COX-2 expression.[106] Also, migration of gastric cancer cells is regulated by the TLR9/NF-κB signaling pathway.[107] Administration of chloroquine, a TLR9 inhibitor, in the human gastric carcinoma cell line MGC803 inhibits cell proliferation and suppresses the expression of COX-2, MMP2, MMP7, and NF-κB p65.[107]

Some *TLR9* gene polymorphisms are associated with gastric cancer. *TLR9* rs5743836 and rs187084 polymorphisms are potential risk factors for gastric cancer progression in a Brazilian population.[108] The *TLR9* rs5743836 polymorphism occurs in the gene promoter associated with elevated TLR9 expression.[98,99] However, this polymorphism is not associated with gastric cancer risk in a Caucasian population.[109] A higher gastric carcinoma risk and poorer survival is associated with a promotor *TLR*9 1486C (rs187084) polymorphism in a Chinese population.[110]

I. Lung Cancer

Lung cancer is one of the most lethal malignancies worldwide. TLR9 is highly expressed in lung carcinoma tissue.[111] Administration of the TLF9 agonist CpG ODN in B cells suppresses the growth of lung tumors by allowing the presentation of antigen and the production of antitumor immunoglobulins. However, increased lung tumor growth is associated with CpG ODN administration in a B cell–null mouse model, which exhibits an immune-suppressive environment.[111] CpG ODN stimulates the release of VEGF, which, because of the formation of

new vessels, worsens lung tumor lesions.[112] CpG ODN also increases the expression of IL-6, activation of STAT3, and cell proliferation in lung tumor–bearing mice.[112] The level of mtDNA is markedly increased in lung cancer patients compared with healthy individuals and plays a role in lung cancer progression and metastasis.[113] ODN M362 is a synthetic CpG-rich sequence that act as a TLR9 agonist. ODN fM362 administration in lung cancer cell lines (i.e., A549 and HCC827) promotes the expression of TLR9 and its adaptor protein MyD88, resulting in increased production of IL-8, which plays a key role in tumor invasion, proliferation, angiogenesis, and migration.[113] TLR9 is expressed by mononuclear cells in human patients and a mouse model of lung cancer associated with increased angiogenic factors and poor survival.[114] TLR9 and its signaling inhibition mediated by microRNA7 overexpression reduces lung cancer cell growth and metastasis via regulation of the PI3K regulatory subunit 3/Akt pathway.[110]

The binding of TLR9 with DNAzyme activates the downstream signaling molecule p38 kinase, which stimulates apoptosis of epidermal growth factor receptor–mutated lung cancer cells.[115] DNAzyme is a molecule designed to inhibit the expression of mutant epidermal growth factor receptor in cancer cells and suppress the development of non-small-cell lung cancer. Lefitolimod (MGN1703), a synthetic DNA-based TLR9 agonist, is a potential treatment for small-cell lung cancer patients.[116] Lefitolimod acts by promoting monocyte activation and IFN-γ-induced protein-10 production.[116]

Many studies show that combined treatment of a CpG agonist and another agent is a promising strategy for lung cancer therapy. For instance, one study reports that activation of TLR9 by DV281 is achieved through an inhaled aerosolized therapeutic agent combined with an inhibitor of antiprogrammed cell death protein-1.[117] This combination promotes the response of $CD4^+$ and $CD8^+$ T cells, formation of tertiary lymphoid structures, DC expansion, $CD8^+$ T cell infiltration, and antibody production in non-small-cell lung cancer.[118] Also, the effect of radiofrequency ablation on $CD8^+$ cytotoxic T lymphocyte (CTL) response stimulation, primary tumor growth, and lung metastasis reduction is enhanced by administration of a TLR9 agonist.[119] Activation of TLR9 by CpG ODN 7909 increases the apoptotic effects of radiofrequency ablation on lung cancer cells by increasing expression and phosphorylation of cellular tumor antigen p53 protein, genome polyprotein, MAPK14, and B cell lymphoma 2-associated X protein (Bax), and by downregulating Bcl-2 expression.[120]

J. Ovarian Cancer

TLR9 is expressed in normal and cancerous human ovarian tissues.[121,122] The level of TLR9 expression is higher in human ovarian cancer tissue than in normal ovarian tissue.[121] The elevation of TLR9 expression in ovarian tumors is associated with greater cancer cell differentiation, more advanced FIGO (International Federation of Gynaecological Oncologists) stage, and advanced lymph node metastasis.[121] Also, TLR9 expression is positively correlated with ovarian tumor grade, ovarian cancer cell migration, and increased NF-κB activation.[123] Hypoxia-induced ovarian cancer stimulates the expression of TLR9 and increases TLR9 ligand release by the human ovarian cancer cell line SKOV3.[124] Synthetic CpG ODN 2006 administration reduces cancer cell sensitivity to cisplatin, a chemotherapy agent used to reduce ovarian cancer growth.[124]

On the other hand, TLR9 makes a positive contribution by prolonging survival.[122] Also, CpG ODN has antitumor effects against ovarian cancer, which are enhanced when combined with the antimicrobial peptide LL-37. This combination results in greater survival of mice as well as increased NK cell activity and proliferation in mice bearing ovarian cancer.[125] In a mouse model, CpG ODN in combination with cisplatin or cetuximab increases median survival time.[126] A mechanism that might explain the anticancer effect of CpG ODN is the suppression of DNA repair gene expression in tumors. CpG ODN improves the effect of cisplatin on DNA damage and enhances animal survival.[127]

K. Pancreatic Cancer

Inflammation and the tumor microenvironment are major contributors to pancreatic cancer

pathogenesis. Compared with normal pancreas tissue, pancreatic cancer tissue shows elevated TLR9 expression, which plays a role in cancer cell invasion and metastasis.[128] TLR9 works as a protumorigenic factor in pancreatic carcinoma, and its deletion exerts protective effects and prolongs survival in an animal model.[129] TLR9 ligands are highly present in the tumor microenvironment and are correlated with increased TLR9 expression during pancreatic oncogenesis.[129] Additionally, activation of TLR9 increases autoregulative growth and proliferation of human pancreatic cancer cells.[130] Stimulation of TLR9 signaling is involved in the activation of MAPK and the expression of both VEGF/platelet-derived growth factor and the antiapoptotic molecule Bcl-xL.[130]

By contrast, administration of the TLR9 agonist CpG ODN 1826 promotes the ability of the immune stimulatory complex, an antitumor vaccine, to activate the T cell response to tumor antigens and induce tumor regression in an animal model. This combination also promotes cancer cell death and animal survival.[131] Similarly, prolonged survival is correlated with the expression of TLR9 in pancreatic ductal adenocarcinoma specimens, and downregulation of TLR9 expression is an independent risk factor for pancreatic cancer-related mortality.[132] Co-administration of the TLR9 agonist IMO and cetuximab reduces pancreatic cancer growth *in vivo* and *in vitro* by suppressing cancer cell survival and inhibiting phosphorylation of MAPK.[93]

L. Prostate Cancer

Prostate cancer tissue expresses higher TLR9 than control tissue.[133–135] Increased TLR9 expression is associated with a greater probability of biochemical recurrence.[133] TLR9 can promote prostate cancer cell invasiveness via MMP-13 stimulation.[134] Furthermore, the TLR9 agonist CpG ODN stimulates the expression of COX-2 through NF-κB activation, which promotes tumor metastasis and invasion.[136] Low progression-free survival is associated with expression of TLR9 in cancer cells.[137] Silencing TLR9/STAT3 activity via *STAT3* siRNA reduces myeloid-derived suppressor cell-regulated immunosuppression in prostate cancer.[138] Additionally, TLR9 plays an essential role in the propagation and self-renewal of prostate cancer cells *in vivo* by increasing the expression of proinflammatory and stem cell–related biomarkers.[139] Administration of CpG-*RELA* siRNA or CpG-*STAT3* siRNA in $TLR9^+$ prostate cancer cells suppresses their growth and clonogenic potential.[139] TLR9 silencing inhibits PC-3 invasion and migration by regulating signaling involving MMP2, MMP9, IL8, and chemokine receptor-4.[140] The anticancer effects of nobiletin, an O-methylated flavonoid, is mediated by inhibition of TLR9/nIRF7 and TLR4/TRIF/IRF3 signaling pathways, resulting in the reduction of cancer cell growth and secretion of IFN-α and IFN-β.[141] However, administration of CpG enhances the therapeutic efficiency of the ISCOMATRIX cancer vaccine in TRAMP-C1 prostate cancer mouse models by promoting the response of $CD8^+$ T cells.[142]

No correlation between the *TLR9* gene polymorphism G2848A (rs352140) and prostate cancer susceptibility was found in a North Indian population.[143] Table 1 shows the association of *TLR9* SNPs with human cancers.

M. Other Cancers

Melanoma is a potentially fatal skin cancer that develops from melanocytes, which produce pigments. TLR9 is expressed by cutaneous malignant melanoma.[144] One study examined the antitumor activity of TLR9 in melanoma after intralesional injection of a TLR9 agonist, PF-3512676, in melanoma patients. PF-3512676 promoted IL-6, IFN-γ induced protein-10, IL-12, p40, and TNF-α expression in cutaneous or subcutaneous metastatic melanoma lesions that are associated with moderate to abundant lymphocyte infiltration.[145] Treatment with Trp2, a peptide vaccine, along with CpG OND, exerts antitumor effects against melanoma and increases Ag-specific IFN-γ and $CD8^+$ CTL responses.[146] Co-administration of dacarbazine, a chemoimmunotherapeutic agent, with CpG ODN induces T cell immune response, reduces tumor growth, and enhances survival time in a mouse

TABLE 1: Association of TLR9 SNPs with human cancers

Polymorphism	Cancer	Ethnicity	Effects	Refs.
rs187084	Liver	Spanish	Lower HCC recurrence	44
	Cervical	Indian	Increased HPV infection; increased cancer risk	71
		Caucasian	Increased cancer risk	72
		Mixed	Decreased cancer risk	72
	Colorectal	Greece	Increased metastatic disease; reduced survival	98
	Gastric	China	Increased carcinoma risk; poor prognosis	110
rs352140	Breast	American	Increased cancer risk	63
	Cervical	Caucasian	Increased cancer risk	72
rs5743836	Colorectal	Greece	Increased metastatic disease; reduced survival	98
rs352139	Colon	Saudi	Increased cancer risk	99
rs352144	Colon	Saudi	Increased cancer risk	99

malignant melanoma model.[147] In addition, CpG conjugated with carbon nanotubes, a novel carrier system, improves antitumor activity in a mouse model.[148]

Esophageal cancer causes high mortality. Compared with normal esophageal tissue, TLR9 is highly expressed in esophageal squamous dysplasia and squamous cell carcinoma.[149] An increase in TLR9 expression in squamous cell carcinomas is associated with higher tumor grade and lymph node and distant metastasis.[150] TLR9 expression is positively correlated with tumor size, stage, and location.[151] TLR9 inhibition via chloroquine significantly abrogates invasion of the esophageal cancer cell line TE10, which is reversed by CpG ODN. This TLR9 agonist promotes the gene expression of COX-2, MMP-2, MMP-7, and COX-2 as well as the activation of NFκB signaling.[151]

As the incidence of renal cancer and its mortality is growing rapidly worldwide, studies of the role in TLR9 in renal cell carcinoma are needed. TLR9 is expressed by both normal kidney tissue[152] and renal cell carcinoma.[153] TLR9 expression is associated with a longer survival time, and a lack of TLR9 is associated with poor prognosis.[153]

Human bladder cancer cells express TLR9. Activation of TLR9 by CpG ODN decreases cancer cell viability and increases cell invasion. Also, TLR9 agonist administration prompts production of the angiogenic factors INF-β, IL-8, and TNF-α[154] (Table 2).

TABLE 2: Observed changes in TLR9 expression in human cancers

Cancer	TLR9 expression
Liver	Increased[45]
Brain	Increased[45,46]
Breast	Increased[53,54]
Cervical	Increased[66–68]
CLL	Increased[75]; decreased[76]
Lymphoma	Increased[81]
Colorectal	Decreased[91]
Gastric	Increased[100]
Lung	Increased[111]
Ovarian	Increased[121]
Pancreatic	Increased[128]
Prostate	Increased[133–135]
Esophageal	Increased[149]

VII. CONCLUSION

Preclinical and clinical studies of TLR9 features, ligand recognition, isoforms, signaling, and critical roles in inflammatory and cancer-associated diseases have progressed remarkably. However, the biological characteristics of this receptor in health and disease are not fully understood. The regulatory mechanisms that mediate TLR9 and adaptor interactions must be clarified. TLR9 downstream signals are mediated by MyD88, MAL, and SCIMP, but further downstream signals of TLR9 through MAL and SCIMP adaptors have not been identified. Although TLR9 is known to have five isoforms, their cellular functions in various tissues and organs and possible roles in disease remain to be investigated. Also, further studies are needed to understand the mechanism and regulators of TLR9 trafficking.

The impact of TLR9 expression in cancer tissue is poorly understood but is associated with both positive and negative outcomes. TLR9 is highly expressed in most human cancers and is recognized as an important factor in cancer cell growth, invasion, survival, and metastasis. Understanding the relationship between TLR9 and the tumor microenvironment is needed to further understand the role of TLR9 in cancer cell behavior. However, accumulating evidence suggests that TLR9 agonists are promising therapeutic agents for certain cancers. Even so, their mechanisms of action must be clarified to obtain maximum therapeutic efficacy. Co-administration of TLR9 agonists and traditional cancer treatments (i.e., radiation or chemotherapy) can be highly efficacious and beneficial. Further studies of the associations between TLR9 polymorphisms and risk of cancer in different ethnicities are urgently needed. Finally, the role of TLR9 adaptors in cancer should be considered in future studies.

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