

TLR9: A Double-Dealing Toll-Like Receptor

Megan Nielsen¹, Daisuke Nishizaki^{2,*}, Shumei Kato^{2,*}, Razelle Kurzrock¹

¹Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, USA; ²Moore's Cancer Center, University of California San Diego, La Jolla, CA, USA

*These authors contributed equally to this work

Correspondence: Megan Nielsen, Medical College of Wisconsin, 8701 W Watertown Plank Road, Milwaukee, WI, 53226, USA, Email mnien1@mcw.edu

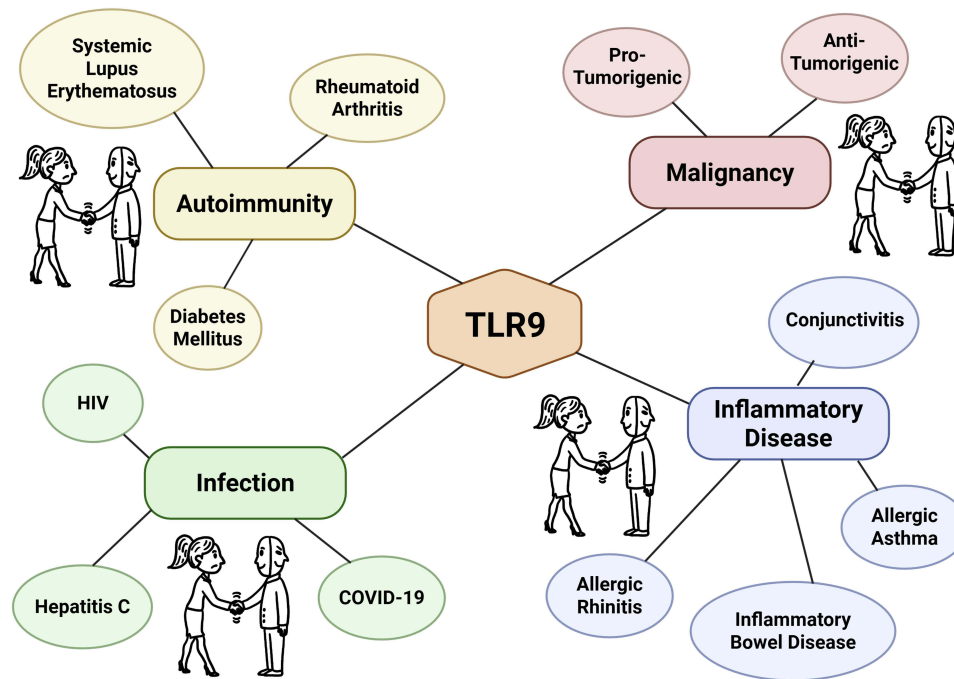
Abstract: In 2011, the Nobel Prize was awarded for the discovery of Toll-like receptors (TLRs) and their critical role in immunity. TLR9 is a key pattern recognition receptor that detects unmethylated cytosine-phosphate-guanine (CpG) DNA motifs, triggering innate and adaptive immune responses against pathogens and damaged host cells. Upon activation, TLR9 triggers signaling cascades that drive NF- κ B, STAT3, and MAPK pathways, modulating inflammatory responses, cellular survival mechanisms, and immune regulation. While TLR9 activation is essential for immune defense, TLR9 acts as a double-dealing TLR in multiple pathologies, including cancer, autoimmunity, and chronic inflammatory disease. In cancer biology, TLR9 exhibits context-dependent roles, acting as a driver of tumorigenesis, a suppressor of tumor growth, and a regulator of immune responses, depending on the tumor type, signaling pathway, and microenvironment. It promotes tumorigenesis in leukemias, gliomas, and cancers of the prostate, bone, lung, and gastrointestinal tract, yet displays tumor-suppressive effects in triple-negative breast cancer, renal cell carcinoma, and virally-associated malignancies, through a variety of mechanisms. Clinically, synthetic CpG oligodeoxynucleotides (ODNs), which function as TLR9 agonists, have emerged as a promising approach in cancer immunotherapy, particularly in combination with other potent anticancer therapies. However, the dual nature of TLR9 signaling poses challenges for therapeutic applications. Its context-dependent effects contribute to inconsistent clinical outcomes and raise concerns about safety and toxicity. This review examines the immunologic function and signaling mechanisms of TLR9, with a focus on its complex, context-specific, and “double-dealing” roles in cancer pathogenesis and therapy.

Keywords: toll-like receptor 9, NF- κ B, STAT3, TLR9 agonist, cancer, immunotherapy, inflammation

Introduction

The 2011 Nobel Prize in Physiology and Medicine was awarded to Bruce Beutler, Jules Hoffmann, and Ralph Steinman for their discoveries regarding immune system function, especially Toll-like receptors (TLRs).¹ The TLR family of pattern recognition receptors (PRRs) identifies pathogen-associated molecular patterns (PAMPs) from invading microbes and damage-associated molecular patterns (DAMPs) from stressed or damaged host cells.^{2,3} TLRs can be broadly categorized by their cellular localization and ligand types. Cell-surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6) primarily sense lipid- and protein-based microbial components, whereas endosomal TLRs (TLR3, TLR7/8, TLR9) detect nucleic acids.⁴ Among the nucleic acid-sensing TLRs, TLR9 is unique in both its ligand specificity and signaling pathways. TLR9 is constitutively expressed in both plasmacytoid dendritic cells (pDCs) and B cells and primarily recognizes unmethylated cytosine-phosphate-guanosine (CpG) DNA-motifs that are common among bacteria and single-stranded DNA viruses, but rare, and usually methylated, in mammalian DNA.⁵ TLR9 signals through a myeloid differentiation 88 (MyD88)-dependent pathway to activate nuclear factor kappa B (NF- κ B), activator protein 1 (AP-1), and a broad interferon regulatory factor (IRF) repertoire—including IRF-1, IRF-5, IRF-7, and IRF-8—stimulating both type I interferons (IFN) and pro-inflammatory cytokines.⁶ In contrast, TLR3 detects dsRNA and signals exclusively through the TIR-domain-containing adaptor-inducing beta interferon (TRIF) adaptor to activate a predominantly antiviral response.^{4,6} TLR7 and TLR8 signal through MyD88 and, like TLR9, can activate NF- κ B and AP-1 pathways; however,

Graphical Abstract



they recognize ssRNA and are classically associated with an acute antiviral type I IFN response that differs from the broader range of signaling elicited by CpG DNA.^{6,7}

The outcome of TLR9 activation depends on the nature and origin of its ligands, which include both exogenous microbial DNA and endogenous host-derived DNA. Exogenous CpG-rich DNA derived from bacteria and viruses are internalized by antigen presenting cells (APCs), including pDCs and B cells, and routed to endosomal compartments, where they drive a robust IRF-7-dependent type I IFN response to combat the pathogen.⁶ Exogenous TLR9 activation also plays a role in cancer, as several tumor types—including esophageal, gastric, colorectal, and pancreatic cancers—are exposed to bacterial CpG DNA that can activate TLR9 and contribute to chronic inflammation and tumorigenesis.^{8–10} In contrast, endogenous TLR9 ligands arise from nuclear and mitochondrial DNA (mtDNA) released during necrosis, apoptosis, hypoxia, oxidative stress, or cytotoxic therapy and preferentially activate tissue-resident cells, including epithelial, parenchymal, and stromal cells. Host DNA is typically methylated and therefore a poor activator of TLR9; however, many tumors exhibit profound genomic hypomethylation and release cell-free, unmethylated, CpG-rich DNA fragments that can activate TLR9 and drive pro-tumorigenic inflammation.^{11–14} Endogenous methylated CpG sequences may also activate TLR9 when delivered within extracellular vesicles or apoptotic bodies—structures that bypass the methylation barrier by physically trafficking DNA into endosomal compartments—although this delivery generally produces weaker, NF- κ B-dominant signaling with minimal type I IFN production.^{6,15} Host mtDNA released during cell damage or stress is largely unmethylated and CpG-rich, capable of efficiently engaging TLR9 and contributing to tumorigenesis in many cancer types.^{16–19}

Once CpG binding has occurred, TLR9 initiates a well-defined MyD88-dependent signaling cascade that stimulates both innate and adaptive immune responses.² CpG binding recruits MyD88, MyD88 adaptor-like (MAL), and Src kinase-interacting membrane protein (SCIMP), forming a Myddosome complex with interleukin-1 receptor-associated kinase-1 and -4 (IRAK1, IRAK4).³ This complex recruits TNF receptor-associated factor-6 (TRAF6), which induces interferon regulatory factor 7 (IRF-7) activation and transforming growth factor- β -activated kinase 1 (TAK1) phosphorylation, leading to mitogen-activated protein kinase (MAPK)-dependent AP-1 activation and NF- κ B translocation via inhibitor of NF- κ B (I κ B) phosphorylation

(Figure 1).^{2,5} NF- κ B, AP-1, and IRF-7 mediate TLR9-induced inflammation by promoting the transcription of proinflammatory cytokines, the production of costimulatory molecules, and the activation of adaptive immune cells.^{2,5} While TLR9 signaling is crucial for adequate immune responses to pathogen invasion, excessive TLR9 activation, resulting from TLR9 overexpression or CpG demethylation, can lead to inflammatory and neoplastic pathologies.³ This review will outline the involvement of TLR9 in tumor progression, immune modulation, novel cancer therapy strategies, and diseases beyond cancer.

TLR9 Pathways in Cancer

TLR9 has a complex, context-dependent role in tumorigenesis, acting as both a stimulator of tumorigenesis and a promoter of antitumor immunity, depending on the cell type, tumor stage, and tumor microenvironment (TME).

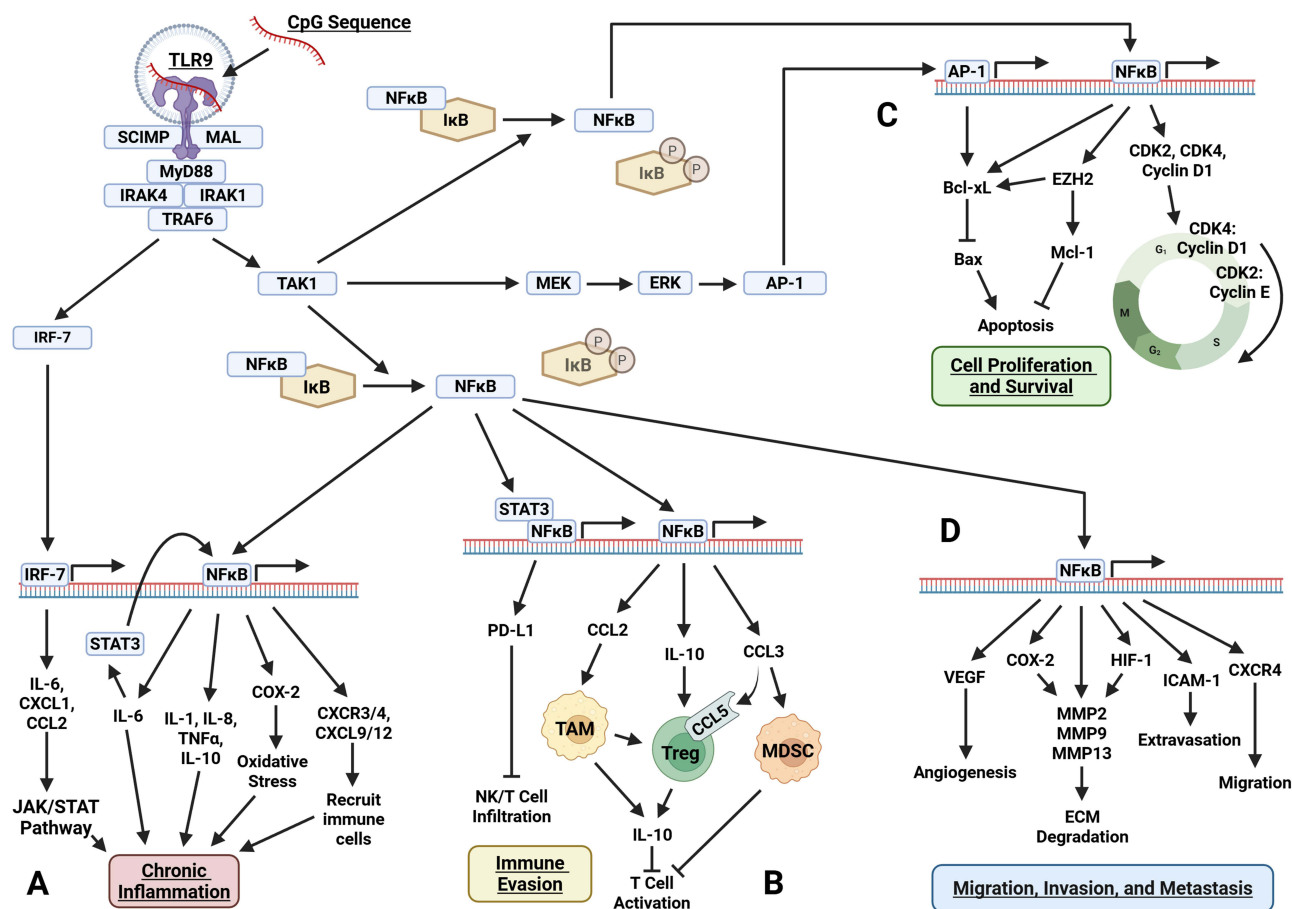


Figure 1 Pro-tumorigenic pathways activated by TLR9 in tumor cells. Upon CpG sequence recognition, TLR9—via MyD88 signaling—induces downstream activation of NF κ B, AP-1, and IRF-7. These transcription factors drive major tumor-promoting mechanisms including (A) chronic inflammation via induction of STAT3, IL-6, IL-8, IL-10, TNF α , COX-2, and chemokines; (B) immune evasion through STAT3/NF κ B-mediated PD-L1 expression and recruitment of TAMs, Tregs, and MDSCs; (C) cell proliferation and survival through upregulation of cyclins, CDKs, and anti-apoptotic proteins; and (D) migration, invasion, and metastasis through VEGF, HIF-1, ICAM-1, CXCR4, and MMPs. Figure created with BioRender.

Abbreviations: AP-1, Activator Protein 1; Bax, BCL2-Associated X Protein; Bcl-xL, B-Cell Lymphoma Extra Large; CCL2, C-C Motif Chemokine Ligand 2; CCL3, C-C Motif Chemokine Ligand 3; CCL5, C-C Motif Chemokine Ligand 5; CDK2, Cyclin-Dependent Kinase 2; CDK4, Cyclin-Dependent Kinase 4; COX-2, Cyclooxygenase-2; CXCL1, C-X-C Motif Chemokine Ligand 1; CXCL9, C-X-C Motif Chemokine Ligand 9; CXCL12, C-X-C Motif Chemokine Ligand 12; CXCR3, C-X-C Chemokine Receptor Type 3; CXCR4, C-X-C Chemokine Receptor Type 4; ECM, Extracellular Matrix; ERK, Extracellular Signal-Regulated Kinase; EZH2, Enhancer of Zeste Homolog 2; HIF-1, Hypoxia-Inducible Factor 1; ICAM-1, Intercellular Adhesion Molecule 1; I κ B, Inhibitor of Nuclear Factor Kappa B; IL-1, Interleukin-1; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-10, Interleukin-10; IRAK1, Interleukin-1 Receptor-Associated Kinase 1; IRAK4, Interleukin-1 Receptor-Associated Kinase 4; IRF-7, Interferon Regulatory Factor 7; JAK/STAT, Janus Kinase/Signal Transducer and Activator of Transcription; MAL, MyD88-Adaptor-Like; MDSC, Myeloid-Derived Suppressor Cell; MEK, Mitogen-Activated Protein Kinase Kinase; Mcl-1, Myeloid Cell Leukemia 1; MMP2, Matrix Metalloproteinase 2; MMP9, Matrix Metalloproteinase 9; MMP13, Matrix Metalloproteinase 13; MyD88, Myeloid Differentiation Primary Response 88; NF κ B, Nuclear Factor Kappa B; NK Cell, Natural Killer Cell; PD-L1, Programmed Death-Ligand 1; SCIMP, src Kinase-Interacting Membrane Protein; STAT3, Signal Transducer and Activator of Transcription 3; TAK1, Transforming Growth Factor-Beta-Activated Kinase 1; TAM, Tumor-Associated Macrophage; TNF α , Tumor Necrosis Factor Alpha; TRAF6, TNF Receptor-Associated Factor 6; Treg, Regulatory T Cell; TLR9, Toll-Like Receptor 9; VEGF, Vascular Endothelial Growth Factor.

Oncogenic Signaling and Tumor Progression

TLR9 signaling promotes tumorigenesis by driving proliferation, invasion, and immune modulation across multiple cancer types. TLR9 overexpression is correlated with poor outcomes in leukemia, glioma, osteosarcoma, and cancers of the stomach, colon, cervix, and prostate.^{20–26} Its downstream signaling influences tumor initiation, progression, and metastasis, with NF- κ B acting as a central mediator of TLR9-induced oncogenesis.^{23,24} Upon activation, NF- κ B regulates the transcription of proinflammatory cytokines, growth factors, and anti-apoptotic proteins.²⁷ Cross-talk with signal transducer and activator of transcription 3 (STAT3) and AP-1 further amplifies these tumor-promoting effects. TLR9 also engages other central oncogenic pathways, including IRF-7 and MAPK. Collectively, these interconnected signaling cascades mediate TLR9's tumor-promoting activity, driving several key hallmarks of cancer.

Inflammatory Pathways and Tumor Initiation

Early in tumorigenesis, TLR9-induced activation of NF- κ B promotes chronic inflammation and supports cell proliferation and survival, particularly in inflammatory-driven malignancies such as gastric, pancreatic, and colorectal cancer (CRC) (Figure 1A).³ Major inflammatory mediators stimulated by NF- κ B, including interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor- α (TNF- α), amplify its signaling through positive feedback, thereby sustaining a proinflammatory TME.²⁸ In gastric cancer, high TLR9 expression correlates with inflammation severity and marks progression from *H. pylori* gastritis to gastric adenocarcinoma.²⁶ In addition to stimulating proinflammatory cytokines IL-6, IL-8, and TNF α , TLR9-induced NF- κ B activation also upregulates cyclooxygenase-2 (COX-2), facilitating the release of reactive oxygen species (ROS) from malignant adenocarcinoma cells and tumor-associated macrophages (TAMs).²⁹ This process induces oxidative stress and DNA damage, creating an inflammatory TME that accelerates tumor progression. A similar TLR9/NF- κ B-driven COX-2 pathway occurs in CRC, accompanied by the release of chemokine receptors 3 and 4 (CXCR3, CXCR4) and chemokine ligands 9 and 12 (CXCL9, CXCL12) which recruit additional immune cells.^{24,30} This effect is mediated by NF- κ B's interaction with STAT3—a protein that regulates many aspects of cell growth, survival, and differentiation—and is frequently dysregulated in CRC and other tumors.³⁰ NF- κ B cooperatively binds with STAT3 to activate genes that drive tumor-promoting inflammation and cancer cell proliferation, invasion, angiogenesis, and metastasis.²⁸ IL-6, an essential downstream effector of NF- κ B:STAT3 signaling, regulates proliferation, survival, and inflammation and is critical in early CRC tumorigenesis.³⁰ Similarly, in pancreatic cancer, TLR9 activation in tumor cells induces nearby stellate cells to secrete inflammatory cytokines (IL-1, IL-10, TNF- α) and chemokines (CCL3, CCL11, CCL12), contributing to a proinflammatory TME that promotes tumorigenesis.⁸

TLR9 activation is associated with pro-tumorigenic inflammation in many other cancer types as well; however, the exact mechanisms underlying this association are not fully understood. In chronic lymphocytic leukemia (CLL), high TLR9 expression is correlated with increased production of proinflammatory cytokines and chemokines, as well as elevated NF- κ B and STAT3 activity.²⁵ Similarly, TLR9 is upregulated in glioma, where it is associated with advanced histopathological grades, stem cell-like phenotypes, and tumor progression.^{21,31} In these tumors, IRF-7 is a key regulator of type I interferons and a major mediator of pro-tumorigenic inflammation.³¹ Its activation in glioma promotes secretion of IL-6 and chemokine ligands 1 and 2 (CXCL1, CXCL2), activating the Janus kinase/STAT3 pathway and promoting stem cell-like phenotypes and angiogenesis.³¹ Although no studies have specifically examined the effects of TLR9 on IRF-7 signaling in glioma, the known activation of IRF-7 downstream of TLR9 suggests a potential connection between TLR9 expression and IRF-7-mediated inflammation in this context.

Regulation of Cell Proliferation and Survival

TLR9 signaling through NF- κ B, STAT3, and AP-1 promotes cell cycle progression and inhibits apoptosis across many tumor types by inducing expression of cell cycle regulators and upregulating survival genes (Figure 1C). B-cell lymphoma-extra large (Bcl-xL), a central mediator of cell survival, is upregulated in CLL, liver, pancreatic, and colorectal neoplasms.^{24,32–34} Bcl-xL promotes survival by inhibiting the pro-apoptotic factor Bcl-2-associated X protein (Bax), which prevents mitochondrial pore formation and blocks cytochrome c release.²⁷ In CRC, Bcl-xL expression is regulated by NF- κ B, whereas other tumors rely on alternative mechanisms.²⁴ TLR9 signaling in CLL

activates enhancer of zeste homolog 2 (EZH2), an epigenetic regulator that upregulates Bcl-xL and myeloid cell leukemia 1 (Mcl-1), likely by transcriptionally silencing tumor suppressors that normally inhibit their expression.³² This decreases the cleavage of pro-apoptotic factors, including poly (ADP-ribose) polymerase (PARP) and caspase-3, suppressing apoptosis and enhancing cell proliferation.³² In pancreatic cancer, TLR9 regulates Bcl-xL through the MAPK pathway, as evidenced by increased phosphorylation of the MAPK protein extracellular signal-regulated kinase (ERK).³⁴ Similar to pancreatic cancer, TLR9-driven Bcl-xL expression in hepatocellular carcinoma (HCC) occurs independently of NF- κ B and IRF-7, suggesting an alternative mechanism potentially involving MAPK/AP-1 signaling.³³ AP-1 is a downstream effector of TLR9 signaling and is implicated in other tumors, such as oral squamous cell carcinoma (OSCC).³⁵ Given that AP-1 can directly regulate Bcl-xL expression, it is plausible that TLR9 activation increases Bcl-xL activity via AP-1, thereby suppressing apoptosis and promoting tumorigenesis in HCC.

TLR9 signaling promotes cell cycle progression through NF- κ B-mediated activation of key cell cycle proteins. In HR+ breast cancer, osteosarcoma, and cervical cancer, TLR9/NF- κ B signaling upregulates cyclin D1, facilitating the transition from G1 to S phase and promoting cell proliferation.^{23,36,37} TLR9 also increases the expression of cyclin-dependent kinase (CDK) proteins, including CDK4 in cervical cancer and CDK2 in osteosarcoma and lung cancer.^{23,37,38} In OSCC, TLR9 activates AP-1, which binds to the cyclin D1 promoter, enhancing its transcription and promoting cell cycle progression.³⁵

Tumor Migration, Invasion, and Metastasis

Invasion, angiogenesis, and intravasation/extravasation are critical processes driving tumor progression and metastasis.³⁹ Elevated TLR9 expression is linked to enhanced invasion, migration, angiogenesis, and metastasis across various cancers through specific signaling pathways (Figure 1D). In prostate and pancreatic cancer, TLR9 upregulates vascular endothelial growth factor (VEGF), a key regulator of angiogenesis, increasing tumor cell invasion and migration in both cell lines³⁴ and patients.²⁰ NF- κ B mediates this effect by binding to the VEGF promoter and initiating its transcription.^{20,27} VEGF then promotes endothelial cell proliferation, vascular permeability, and vasculogenesis, increasing nutrient delivery to tumor cells.²⁰

Extracellular matrix (ECM) degradation is crucial to tumor invasion and is primarily driven by matrix metalloproteinases (MMPs).³⁹ These proteolytic enzymes are frequently overexpressed in cancer and positively correlated with TLR9 signaling in many tumor types. TLR9 activation induces the release of MMP-2 and MMP-9 in osteosarcoma, glioma, glioblastoma, lung, gastric, and prostate cancer, while MMP-13 contributes to ECM degradation in prostate cancer.^{23,29,40–43} NF- κ B can directly upregulate MMPs²⁷ or act indirectly via COX-2—a proinflammatory mediator that increases MMP expression.⁴⁴ In brain tumors, hypoxia amplifies TLR9-induced MMP expression.⁴⁰ A common hallmark of solid tumors, hypoxia promotes angiogenesis and invasion by activating HIF-1, a transcription factor that upregulates MMPs and further enhances TLR9 signaling in a positive feedback loop that perpetuates tumor invasion.⁴⁰

TLR9 activity in lung and prostate cancer is associated with increased CXCR4 expression, which drives tumor cell migration.^{41,42} To facilitate metastasis, chemokines secreted in blood vessels and distant organs bind to chemokine receptors on tumor cells, directing their migration to distant sites. At these sites, cell adhesion molecules facilitate the attachment and extravasation of tumor cells into new tissues. This occurs in lung cancer, where TLR9 activity increases the expression of intercellular adhesion molecule 1 (ICAM-1).⁴¹ By regulating critical molecules such as VEGF, MMPs, CXCR4, and ICAM-1, TLR9 facilitates tumor invasion, migration, and metastasis. As a result, it is a promising target for therapies aimed at limiting tumor spread in aggressive cancers.

There is evidence to suggest that TLR9 may facilitate tumor progression in part by promoting or sustaining the epithelial-mesenchymal transition (EMT), a key mechanism that enables malignant cells to acquire migratory and invasive phenotypes. In lung adenocarcinoma, EMT induction enhances sensitivity to CpG stimulation, leading to increased cytokine production—an effect that is lost when TLR9 is inhibited.⁴⁵ In breast cancer, high TLR9 expression correlates with more aggressive, invasive subtypes, and EMT-like transcriptional profiles.⁴⁶ For example, TLR9 expression is positively associated with expression of zinc finger E-box-binding homeobox 2 (ZEB2), Snail family transcriptional repressor 1 (SNAIL), twist family bHLH transcription factor 1 (TWIST1), and vimentin.⁴⁶ Additional studies in

cancer models are needed to fully characterize this mechanism; however, TLR9 activation is known to induce EMT in non-malignant inflammatory settings, supporting the plausibility of a similar pathway in tumors.⁴⁷

Immune Modulation and Tumor Immune Evasion

The immune system plays a dual role in tumorigenesis, with distinct immune cell populations either suppressing or supporting cancer development. TAMs, myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) promote tumor progression by producing growth factors, pro-angiogenic molecules, and matrix-degrading enzymes (Figure 1B).³⁹ In CLL, TLR9 increases the secretion of the anti-inflammatory cytokine IL-10 and recruits Tregs, which suppress the proliferation of cytotoxic CD4+ T cells and enable tumor immune escape.⁴⁸ In pancreatic cancer, TLR9-dependent signaling directly recruits Tregs and MDSCs to the TME via secretion of CCL3, which binds to chemokine receptor 5 (CCR5) on Tregs.⁸ Within the TME, Tregs release IL-10, suppressing major histocompatibility complex class II (MHC II) expression and costimulatory signals in antigen-presenting cells, while MDSCs inhibit cytotoxic T cell activation, collectively enhancing immunosuppression and promoting tumor progression.⁸ MDSCs similarly accelerate tumor growth in prostate cancer. In this context, TLR9-induced upregulation of leukemia inhibitory factor, an IL-6 family cytokine, activates and recruits a specific subpopulation of MDSCs, namely polymorphonuclear (PMN)-MDSCs, to the TME.⁴⁹ These PMN-MDSCs trigger STAT3 activation, driving the expression of IL-10 and arginase-1 to sustain an immunosuppressive TME.⁴⁹

Mitochondrial DNA (mtDNA) stress is characterized by the release of mtDNA into the cytosol and is often indicative of oncogenesis.³ In HCC, TLR9 detects cytosolic mtDNA and activates the NF- κ B pathway, leading tumor cells to secrete CCL2, which recruits TAMs.⁵⁰ These TAMs then release IL-10 and chemokine ligands 7 and 22 (CCL17, CCL22) to attract additional immunosuppressive cells and support HCC progression.⁵⁰ Notably, TLR9 activation also promotes immune escape in both HCC and OSCC by STAT3-mediated upregulation of programmed cell death ligand 1 (PD-L1) on tumor cells.^{51,52} This upregulation reduces the infiltration and activation of NK cells and CD4+/CD8+ T cells within the TME while increasing Tregs, collectively contributing to an immunosuppressive TME that supports cancer progression.^{51,52}

Antitumorigenic Pathways

TLR9 signaling can protect against cancer development in a few specific contexts. TLR9 overexpression is associated with improved outcomes in triple-negative breast cancer (TNBC), renal cell carcinoma (RCC), neuroblastoma, and cancers of the cervix, head and neck, oropharynx, and pancreas.^{46,53–58} In this protective role, TLR9 interferes with several key hallmarks of oncogenesis, including tumor initiation, evasion, and immune system dysregulation (Figure 2). In these pathways, TLR9 appears to promote a more balanced and stable inflammatory response, which may help limit tumor growth and progression. However, these antitumorigenic mechanisms may differ based on tumor types and microenvironments.

TLR9 and Viral Oncology

Oncoviruses contribute to the malignant transformation of many tumors, including gynecological, hematological, head and neck, and liver cancers. Effective viral immune responses are crucial in preventing tumorigenesis and are mediated, in part, by PRRs, like TLR9, in organ tissues and nearby immune cells. TLR9 recognizes unmethylated CpG motifs present in viral DNA; however, oncogenic viruses frequently suppress its activity to evade immune detection. For example, TLR9 is downregulated by Epstein-Barr virus (EBV) in oropharyngeal cancer and by E6 and E7 from human papillomavirus 16 (HPV16) in head and neck and cervical neoplasms.^{54,55} This impairs antiviral innate immunity and promotes the growth of transformed epithelial cells (Figure 2C). In head and neck cancer, restoring TLR9 expression stabilized p16, a CDK4 inhibitor, which prolonged the S phase and activated cell cycle checkpoints, ultimately reducing cell proliferation and colony formation.⁵⁴ This may explain why low TLR9 expression is associated with higher tumor grades in cervical cancer.⁵⁹ In contrast, other research has instead linked high TLR9 expression with more advanced cervical cancer, so further research is needed to understand TLR9's role in this malignancy.²² In early-stage ovarian tumors, high TLR9 expression is associated with extended patient survival; however, the underlying mechanism for this

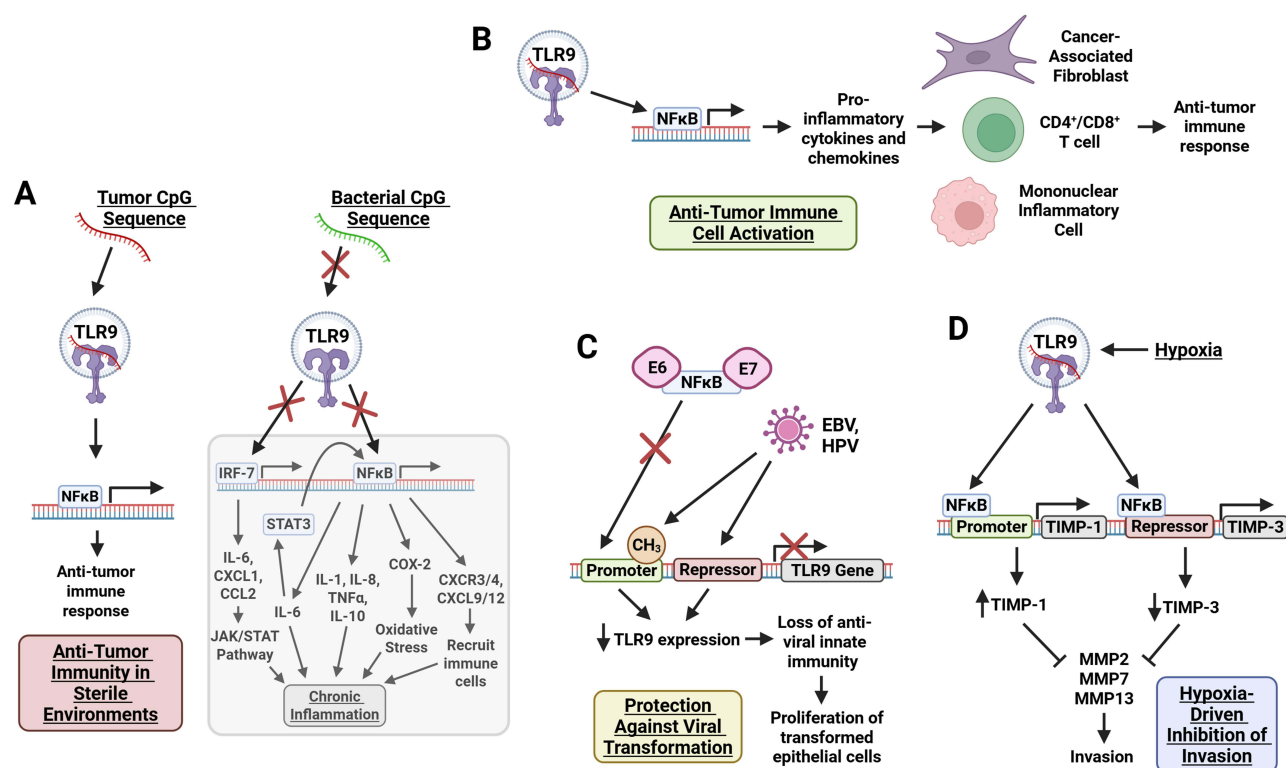


Figure 2 Anti-tumorigenic roles of TLR9 in pre-cancerous cells. Upon CpG sequence recognition, TLR9 activates NFκB through MyD88-dependent signaling, driving transcriptional regulation that (A) supports anti-tumor immunity in sterile environments; (B) sustains innate immune defenses to protect against viral transformation; (C) modulates the expression of hypoxia-sensitive invasion suppressors, TIMP-1 and TIMP-3; and (D) promotes anti-tumor immune cell activation through induction of pro-inflammatory cytokines and chemokines. Figure created with BioRender.

Abbreviations: CCL2, C-C Motif Chemokine Ligand 2; CD4+/CD8+, Cluster of Differentiation 4+/8+ T Cells; COX-2, Cyclooxygenase-2; CpG, Cytosine-phosphate-Guanine; CXCL1, C-X-C Motif Chemokine Ligand 1; CXCL9, C-X-C Motif Chemokine Ligand 9; CXCL12, C-X-C Motif Chemokine Ligand 12; CXCR3/4, C-X-C Chemokine Receptor Type 3/4; EBV, Epstein-Barr Virus; E6/E7, Oncoproteins of Human Papillomavirus (HPV); HPV, Human Papillomavirus; IL-1, Interleukin-1; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-10, Interleukin-10; IRAK1, Interleukin-1 Receptor-Associated Kinase 1; IRAK4, Interleukin-1 Receptor-Associated Kinase 4; IRF-7, Interferon Regulatory Factor 7; iNOS, Inducible Nitric Oxide Synthase; JAK/STAT, Janus Kinase/Signal Transducer and Activator of Transcription; MAL, MyD88-Adaptor-Like; MMP2, Matrix Metalloproteinase 2; MMP7, Matrix Metalloproteinase 7; MMP13, Matrix Metalloproteinase 13; MyD88, Myeloid Differentiation Primary Response 88; NFκB, Nuclear Factor Kappa B; SCIMP, src Kinase-Interacting Membrane Protein; STAT3, Signal Transducer and Activator of Transcription 3; TIMP-1, Tissue Inhibitor of Metalloproteinases 1; TIMP-3, Tissue Inhibitor of Metalloproteinases 3; TLR9, Toll-Like Receptor 9; TNFα, Tumor Necrosis Factor Alpha; TRAF6, TNF Receptor-Associated Factor 6.

observation has not been investigated.⁵⁶ The presence of DNA from HPV, EBV, and cytomegalovirus has been detected in ovarian malignancies, suggesting a potential viral contribution to ovarian carcinogenesis.⁶⁰ Given this, TLR9 may play a similar antitumor role in ovarian cancer as it does in cervical and head and neck cancers.

TLR9 in Tumors with Minimal Microbial Exposure

Upregulation of TLR9 is associated with improved survival in patients with pancreatic cancer.⁵⁷ This finding aligns with *in vitro* studies demonstrating that TLR9 signaling suppresses pancreatic cancer migration, invasion, and metastasis.⁶¹ Although the underlying mechanism is unclear, one hypothesis suggests that the relatively sterile environment of the pancreas—compared to bacterial-rich organs such as the oral cavity, stomach, and colon—may allow TLR9 to promote antitumor immune responses without triggering the proinflammatory signaling pathways that drive tumorigenesis in tissues more frequently exposed to microbiota (Figure 2A).⁵⁷ Similar associations between TLR9 expression and favorable prognostic factors in tumors from other relatively sterile organs, such as the kidney and brain, support this hypothesis. In RCC, high TLR9 expression correlates with improved survival, and in neuroblastoma, it inversely correlates with disease stage.^{53,58} However, other tumor-intrinsic mechanisms may be involved as this process cannot fully explain the observed decrease in pancreatic tumor cell proliferation, invasion, and metastasis in tumor cell cultures that lack immune cells. Furthermore, in mucoepidermoid salivary gland carcinoma, high TLR9 expression is associated with less invasion and better survival despite this tumor developing in the oral cavity, an area rich in bacteria.⁶² These

observations suggest that TLR9 exerts context-dependent antitumor effects through both immune-mediated and tumor-intrinsic mechanisms.

Hypoxia-Driven Modulation of Invasion Pathways

The antitumor effects of TLR9 are context-dependent, especially in TNBC, where hypoxic conditions significantly influence TLR9's activity. Although TLR9 is generally upregulated in TNBC, a subset of TNBC patients with low TLR9 expression exhibit significantly decreased survival, suggesting a protective role for TLR9 in this setting.⁴⁶ The ability of hypoxia to promote tumor cell invasion in TNBC depends on TLR9 expression. When TLR9 expression is low, hypoxia enhances invasion through transcriptional inactivation of tissue inhibitor of metalloproteinase 3 (TIMP-3) and downregulation of TIMP-1 and TIMP-2.⁶³ This leads to increased activity of MMP-2, MMP-7, and MMP-13, resulting in unchecked proteolytic activity and increasing tumor cell invasion (Figure 2D). In contrast, TNBC cells with normal or high TLR9 expression respond differently: hypoxia downregulates TIMP-3 without inactivating it, upregulates TIMP-1, and has no effect on TIMP-2.⁶³ This balance in MMP activity restricts tumor cell invasion and may explain the increased survival observed in TNBCs with higher TLR9 expression. Notably, less aggressive subtypes, such as ER+ breast cancer, do not exhibit this trend, likely due to tumor-specific differences in TLR9 activity and downstream signaling under hypoxic conditions.⁶³

Although differences in TLR9 expression correlate with invasion in TNBC, the minimal or absent expression of TLR9 in the low-TLR9 subtype of TNBC makes it unlikely that TLR9 itself directly mediates the hypoxia-induced loss of TIMP-3. Instead, the absence of TLR9 signaling may permit alternative pathways to drive invasion. TLR9 is known to inhibit the signaling of other endosomal TLRs. Chloroquine—an inhibitor of endosomal acidification and TLR activation—reduces invasion in TNBC cells, suggesting that other TLRs on the endosomal membrane may induce pro-invasive phenotypes.⁶³ Therefore, loss of TLR9 in TNBC may allow other endosomal TLRs to promote invasion through TIMP-3 inactivation.⁶³ However, this hypothesis requires experimental validation.

Immune Activation and Tumor Suppression

Although TLR9 expression in tumor cells appears to induce tumor-promoting immune responses, it may also facilitate antitumor immunity in specific contexts and cancer types. For example, TNBC tumors with high TLR9 expression exhibit an inflammatory TME that correlates with improved prognosis.⁴⁶ TLR9-induced activation of NF- κ B drives the expression of proinflammatory cytokines and chemokines, which recruit cancer-associated fibroblasts, mononuclear inflammatory cells, and CD4+/CD8+ T cells (Figure 2B). Importantly, in this context, immune cells are directed against the tumor, thereby strengthening antitumor immunity and contributing to improved outcomes.⁴⁶ Interestingly, TLR9's antitumor immune effects may be particularly relevant following cancer treatment, as in vivo responses to doxorubicin chemotherapy in TNBC depend on TLR9 expression status.^{14,64}

High TLR9 expression also correlates with increased survival in RCC. Given the immunogenic nature of RCC, TLR9's role in antitumor immunity may explain this observation, but this mechanism has not been investigated.⁵⁸ The specific immune populations mediating TLR9's effects in TNBC and RCC require further exploration. However, CD8+ T cells are unlikely to be the primary immune cells involved, as TLR9 expression does not correlate with tumor-infiltrating CD8+ T cell count in either TNBC or RCC.⁶⁴

Immune Cell TLR9 Expression in Tumor Progression and Defense

Given TLR9's involvement in both innate and adaptive immunity, its signaling in immune cells can exert either tumor-promoting or tumor-suppressive effects, depending on the context. Evidence largely supports TLR9's anticancer effects, primarily mediated by pDC and B cells—the only two cell types that constitutively express TLR9. TLR9 signaling in pDCs triggers IFN secretion, which upregulates costimulatory cluster of differentiation 80/86 (CD80/86) molecules on APCs—including pDCs and macrophages—thereby promoting activation of CD4+ T cells (Th1), CD8+ T cells, and NK cells to support antitumor immunity (Figure 3A).⁶⁵ Additionally, pDCs secrete TNF-related apoptosis-inducing ligand (TRAIL), which binds to death receptors on tumor cells and initiates apoptosis (Figure 3C).⁶⁶ TLR9 signaling enhances humoral immunity by driving B cell differentiation into plasma cells, increasing antibody secretion, and enabling

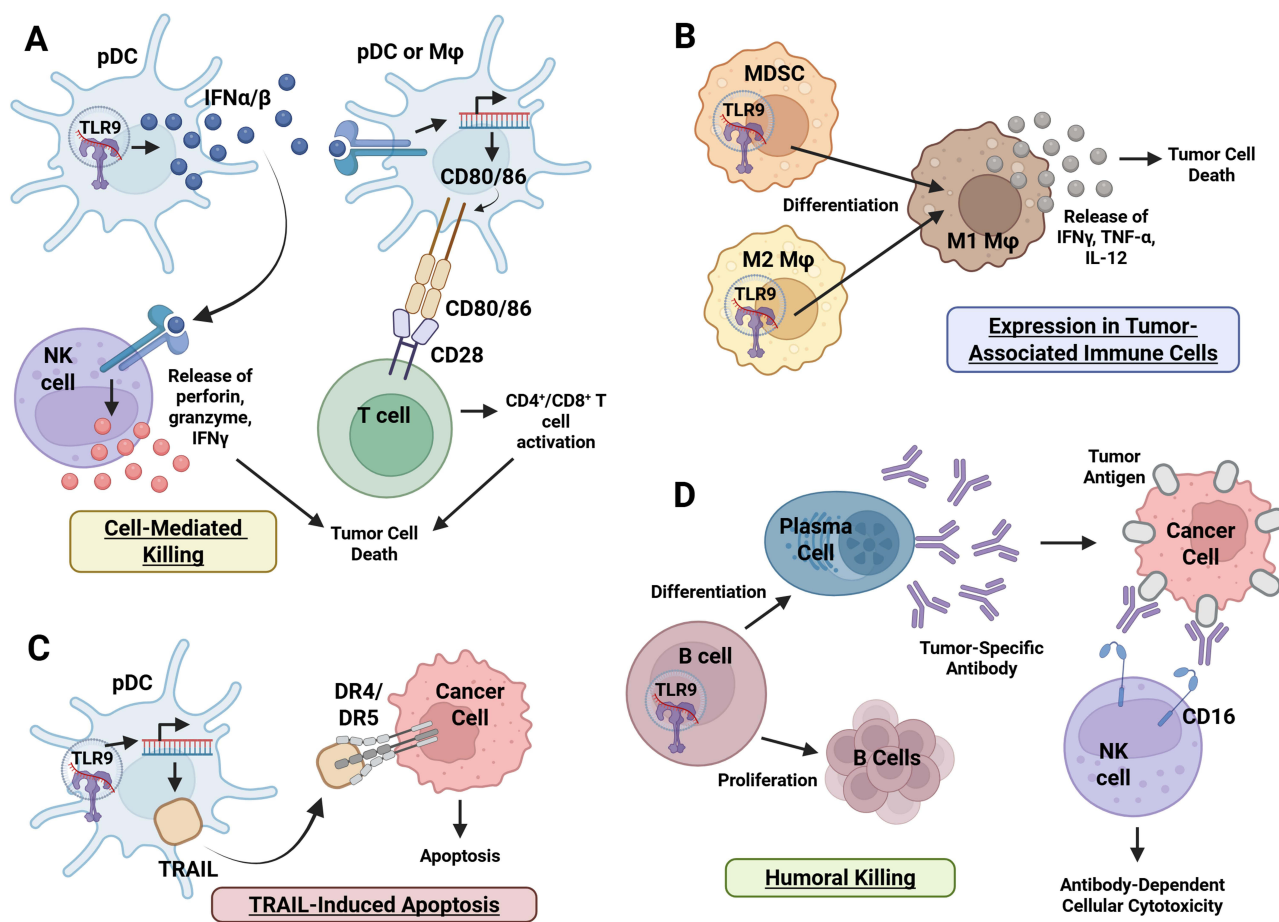


Figure 3 TLR9 expression in immune cells. Upon CpG sequence recognition, TLR9 induces tumor clearance primarily via pDCs, macrophages, and B cells. TLR9 signaling promotes (A) cell-mediated killing through type I IFN-mediated activation of NK- and T-cell cytotoxicity; (B) differentiation of tumor associated immune cells toward a tumoricidal M1 phenotype; (C) TRAIL-induced apoptosis via death ligand expression; and (D) humoral killing driven by B cell proliferation, plasma cell differentiation, and ADCC. Collectively, these responses enable tumor cell apoptosis through innate, cell-mediated, and humoral pathways. Figure created with BioRender.

Abbreviations: ADCC, Antibody-Dependent Cellular Cytotoxicity; CD16, Cluster of Differentiation 16; CD28, Cluster of Differentiation 28; CD4+/CD8+, Cluster of Differentiation 4+/8+ T Cells; CD80/86, Cluster of Differentiation 80/86; CpG, Cytosine-phosphate-Guanine; DR4/DR5, Death Receptor 4/5; IFN α/β , Interferon Alpha/Beta; IFN γ , Interferon Gamma; IL-12, Interleukin-12; M1 M ϕ , M1 Macrophage (Proinflammatory); M2 M ϕ , M2 Macrophage (Anti-inflammatory); MDSC, Myeloid-Derived Suppressor Cell; NK cell, Natural Killer Cell; pDC, Plasmacytoid Dendritic Cell; TLR9, Toll-Like Receptor 9; TNF- α , Tumor Necrosis Factor Alpha; TRAIL, TNF-Related Apoptosis-Inducing Ligand.

antibody-dependent cellular cytotoxicity (Figure 3D).⁶⁵ Immune cell TLR9 signaling exerts antitumor effects across multiple tumor types. For example, TLR9 activation enhances NK and CD8⁺ T cell cytotoxicity in cutaneous T-cell lymphoma (CTCL), drives DC-dependent CD4⁺ T cell responses in melanoma, and promotes DC expansion, CD8⁺ T cell infiltration, and antibody production in lung cancer.^{65,67,68}

Alternatively, because tumor-promoting immune cells (eg, TAMs, MDSCs, and Tregs) contribute to cancer progression, TLR9 signaling within these populations may paradoxically enhance tumor growth. In prostate cancer, TLR9 activation in MDSCs drives STAT3 activation and immunosuppressive effects, inhibiting CD8⁺ T cell proliferation and the secretion of IFN γ - and granzyme-B.⁶⁹ TLR9 expression is elevated in Tregs from patients with head and neck squamous cell carcinoma compared to healthy controls, suggesting that TLR9 may enhance Treg-mediated immunosuppression and tumor immune escape.⁷⁰ Similarly, high TLR9 expression in TAMs correlates with increased tumor recurrence in murine models of melanoma, bladder, and colon cancer.⁷¹

In contrast, other studies suggest that TLR9 expression in tumor-associated immune cells can promote antitumor immunity. In gastric, liver, and colon cancers, TLR9 signaling blocks the immunosuppressive function of MDSCs, restores T cell activity and promotes MDSC differentiation into antitumor macrophages (Figure 3B).^{72–74} TLR9 activation in liver cancer also increases the ratio of M1 (antitumor) to M2 (pro-tumor) TAMs. In melanoma and

neuroblastoma models, TLR9 activates macrophages to induce tumor cell apoptosis via IFN- γ , IL-12, and TNF- α secretion.⁷⁵ Overall, the role of immune TLR9 signaling in cancer is complex and largely dependent on the surrounding TME and immune cells involved.

TLR9 Therapeutics in Cancer

TLR9-based cancer therapies take advantage of the receptor's ability to both activate and regulate the immune system (Tables 1 and 2). The clinical translation of TLR9 modulators has yielded inconsistent results that reflect TLR9's complex, context-dependent biology. Given TLR9's dual role in tumorigenesis, the efficacy of TLR9 agonists or antagonists may depend more on tumor type and microenvironment than on the specific modulator itself. This section examines the clinical applications of TLR9 modulators and analyzes their performance through the lens of TLR9 biology and the molecular pathways discussed earlier.

TLR9 Agonists

Synthetic oligodeoxynucleotides (ODNs) mimic endogenous CpG DNA motifs and induce TLR9 signaling. As mono-therapies, CpG ODNs are insufficient to reverse the immunosuppressive state of most cancers; however, many TLR9 agonists are being explored in combination with other therapies. TLR9 agonists consist of synthetic, single-stranded DNA sequences containing unmethylated cytosine-guanine dinucleotides and are divided into three classes—A, B, and C—based on their structure and immunostimulatory effects.¹⁰⁸ Class A ODNs, including CMP-001, consist of a central palindromic phosphodiester CpG motif and a 3' poly-G-tail containing a phosphorothioate backbone.¹⁰⁸ These CpG agonists predominantly activate pDCs and are selectively retained with MyD88-IRF-7 complexes in pDC endosomes, leading to sustained IRF-7 activation, robust IFN- α secretion, and indirect activation of T and NK cells.^{6,108,109} In melanoma, CMP-001 has been combined with immune checkpoint inhibitors (ICI), pembrolizumab, and nivolumab (Table 2). A Phase II trial combining CMP-001 with nivolumab demonstrated a promising ORR of 45% (14/31 patients);

Table 1 Examples of Clinical Trials Using TLR9 Agonists as Single Agents

Trial ID	Phase	Drug	Mechanism	Cancer Type	ORR ¹ [N/total Patients Treated]	Comments	Ref.	Status
NCT00091208, NCT00043420	I	CpG 7909 (PF-3512676)	Class B CpG ODN	Cutaneous T cell lymphoma	32% [9/28]		[76]	Completed 2007
NCT00043407	I/II	CpG 7909 (PF-3512676)	Class B CpG ODN	Renal cell carcinoma	5% [2/40]		[77]	Completed 2004
NCT00070642	II	CpG 7909 (PF-3512676)	Class B CpG ODN	Melanoma	Not available	CpG 7909: 5/10 patients had measurable responses to more than one melanoma-associated antigen epitope; Saline: 0/11 responses	[78]	Completed 2005
NCT01208194	II	MGN1703	dSLIM	Metastatic colorectal carcinoma	7% [3/43] - MGN1703; 6% [1/16] placebo	Significant PFS improvement in MGN1703 vs placebo group (p<0.05)	[79]	Completed 2013
NCT00729053	II	IMO-2055 (EMD 1201081)	IMO	Renal cell carcinoma	2% [2/89]		Not available	Completed 2008
NCT03052205	Ib	IMO-2125 (tilsolimod)	IMO	Refractory solid tumors	0% [0/35]	Tumor biopsies at 24 hours showed increased immune activation	[80]	Completed 2019
NCT05399654	I/II	TAC-001	TLR9 Agonist Antibody Conjugate	Advanced or metastatic solid tumors	2.9% [2/69]		[81]	Ongoing

¹ORR = CR+PR.

Abbreviations: CpG ODN, Cytosine-phosphate-Guanosine Oligonucleotide; dSLIM, Double-Stem-Loop Immunomodulator; IMO, Immunomodulatory Oligonucleotide; ORR, Objective Response Rate; PFS, Progression-Free Survival.

Table 2 Examples of Clinical Trials Using TLR9 Agonists in Combination with Other Therapies

Trial ID	Phase	Drug	Mechanism	Other Drugs	Cancer Type	ORR ¹ [N/total Patients Treated]	Comments	Ref.	Status
NCT02680184	Ib	CMP-001	Class A CpG ODN	±Pembrolizumab	Metastatic melanoma	19% [30/159] - CMP-001 + Pembro; 17.5% [7/40] - CMP-001		[82]	Completed 2022
NCT03084640	Ib	CMP-001	Class A CpG ODN	Pembrolizumab	Anti-PD-I resistant metastatic melanoma	25% [11/44]		[83]	Completed 2021
NCT03507699	I	CMP-001	Class A CpG ODN	Nivolumab, ipilimumab, radiosurgery	Metastatic colorectal carcinoma	5.3% [1/19]		[84]	Completed 2021
NCT03618641	II	CMP-001	Class A CpG ODN	Nivolumab	Melanoma	45% [14/31]	1 year RFS is 82%	[85]	Completed 2023
NCT00471471	I	CpG 7909 (PF-3512676)	Class B CpG ODN	MART-I, gp100, and tyrosinase vaccine with GM-CSF ²	Metastatic melanoma	9.5% [2/21]	9/20 patients developed reactive CD8 ⁺ T cells against vaccine peptides	[86]	Completed 2011
NCT00070629	II	±CpG 7909 (PF-3512676)	Class B CpG ODN	Taxane/ platinum chemotherapy	Non-small cell lung cancer	38% [28/74] - CpG 7909 + chemo; 19% [7/37] - chemo		[87]	Completed 2007
NCT00254891	III	±CpG 7909 (PF-3512676)	Class B CpG ODN	Taxane/ platinum chemotherapy	Non-small cell lung cancer	28% [114/408] - CpG 7909 + chemo; 23% [96/420] - chemo	Lack of efficacy, safety issue (sepsis)	[88]	Terminated 2008
NCT00321815	II	±CpG 7909 (PF-3512676)	Class B CpG ODN	Erlotinib	Non-small cell lung cancer	10% [7/45] - CpG7909 + erlotinib; 5% [3/39] - erlotinib	No significant difference in PFS	[89]	Completed 2010
NCT00185965	I/II	CpG 7909 (PF-3512676)	Class B CpG ODN	Low dose radiotherapy	Low grade B cell lymphoma	27% [4/15]		[90]	Completed 2010
NCT00880581	II	CpG 7909 (PF-3512676)	Class B CpG ODN	Radiotherapy	B cell lymphoma	23% [7/30]		Not available	Completed 2015
NCT00819806	I	CpG 7909 (PF-3512676)	Class B CpG ODN	Tumor peptides and adjuvant ³	NY-ESO-I expressing tumors	Not available	9/14 patients developed sustained antigen-specific CD8 ⁺ T cell responses	[91]	Completed 2015
NCT00292045	I	±CpG 7909 (PF-3512676)	Class B CpG ODN	NY-ESO-I peptide vaccine	Prostate cancer	0% [0/13]	69% [9/13], 46% [6/13], and 100% [13/13] had antigen-specific CD4 ⁺ , CD8 ⁺ , and humoral responses, respectively	[92]	Completed 2006
NCT00190424	II	±CpG-28 (litenimod)	Class B CpG ODN	SOC (RT + temozolomide)	Malignant glioblastoma	Not available	OS 31% [12/39] - CpG-28 + SOC; OS 26% [11/42] - SOC	[93]	Completed 2010

(Continued)

Table 2 (Continued).

Trial ID	Phase	Drug	Mechanism	Other Drugs	Cancer Type	ORR ¹ [N/total Patients Treated]	Comments	Ref.	Status
NCT00251394	II	ISS-1018	Class B CpG ODN	Rituximab	Refractory follicular lymphoma	48% [11/23]	Average PFS 9 months; increased ADCC and circulating immune cells in the TME	[94]	Completed 2009
NCT01435356	II	AS15 ASCI ⁴	Contains CpG 7909 - class B CpG ODN	recMAGE-A3	Bladder cancer	Not available	OS 35.5 months - recMAGE-A3 + AS15; OS 24.1 months - placebo	Not available	Completed 2017
NCT01425749	I	AS15 ASCI	Contains CpG 7909 - class B CpG ODN	recMAGE-A3	Melanoma	Not available	11/24 patients developed CD4 ⁺ T cell responses; all patients develop Ab responses	[95]	Completed 2015
NCT01149343	I/II	AS15 ASCI	Contains CpG 7909 - class B CpG ODN	recPRAME	Melanoma	Not available	19/33 patients developed CD4 ⁺ T cell responses; all patients develop Ab responses	[96]	Completed 2016
NCT01266603	II	AS15 ASCI	Contains CpG 7909 - class B CpG ODN	recMAGE-A3 and HDIL-2	Melanoma	25% [4/16]		[97]	Completed 2018
NCT01437605	II	AS15 ASCI	Contains CpG 7909 - class B CpG ODN	+recMAGE-A3 ±Poly IC:LC adjuvant	Melanoma	Not available	Treatment ineffective	Not available	Terminated 2018
NCT01380145	I	AS15 ASCI	Contains CpG 7909 - class B CpG ODN	recMAGE-A3 + autologous SCT	Multiple myeloma ⁵	69% [9/13]	13/13 patients developed antigen-specific CD4 ⁺ and humoral responses	[98]	Completed 2014
NCT00952692	I	dHER2 AS15 ASCI	Contains CpG 7909 - class B CpG ODN	Lapatinib	Breast cancer	0% [0/12]	OS 92% at 300 days	[99]	Completed 2012
NCT02266147	I/II	SD-101	Class C CpG ODN	Low-dose radiation	Indolent lymphoma	28% [8/29]		[100]	Completed 2017
NCT03410901	I	SD-101	Class C CpG ODN	BMS986178, RT	Low-grade B-cell lymphoma	7% [1/14]		[101]	Completed 2024
NCT02521870	Ib	SD-101	Class C CpG ODN	Pembrolizumab	Unresectable or metastatic melanoma	78% [7/9] treatment naive; 15% [2/13] PD-1-inhibitor refractory		[102]	Completed 2019
NCT02200081	II	±MGN1703	dSLIM	SOC (platinum-based chemo)	Extensive- stage small cell lung cancer	12% [7/59] - MGN1703 + SOC; 8% [3/37] - SOC	No significant difference in PFS	[103]	Completed 2017

NCT00633529	Ib	IMO-2055 (EMD 1201081)	IMO	Erlotinib and bevacizumab	Non-small cell lung cancer	15% [5/33]		[104]	Completed 2011
NCT01040832	II	±IMO-2055 (EMD 1201081)	IMO	Cetuximab	Recurrent or metastatic squamous cell carcinoma	5.7% [3/53] - IMO- 2055 + cetuximab; 5.7% [3/53] - cetuximab		[105]	Completed 2012
NCT01360827	Ib	IMO-2055 (EMD 1201081)	IMO	5-FU, cisplatin, cetuximab	Recurrent or metastatic squamous cell carcinoma of the head and neck	23% [3/13]	Safety issue (62% [8/13] of patients experienced serious adverse events)	[106]	Terminated 2011
NCT03445533	III	±IMO-2125 (tilsotolimod)	IMO	Ipilimumab	PD-1 inhibitor-refractory advanced melanoma	8.8% [21/238] - IMO-2125 + ipilimumab; 8.6% [21/243] - ipilimumab	Terminated due to lack of efficacy	Not available	Terminated 2021
NCT03684785	Ib/II	AST-008 (cavrotolimod)	Spherical nucleic acid	±Pembrolizumab or cemiplimab	Advanced skin cancer	11.7% [6/51]		[107]	Completed 2022

Notes: ¹ORR = CR+PR. ²MART-1, gp100, and tyrosinase are melanoma-associated antigens commonly included in cancer vaccines and combined with adjuvants like GM-CSF or TLR9 agonists to enhance their immune response. ³Antigen peptides included NY-ESO-1 for all patients, and Melan-A/MAGE-A10 for HLA-A2 positive patients. ⁴AS15 ASCI is a cancer vaccine containing the adjuvant systems AS01B and AS07A; AS01B contains liposomes with 3D-MPL and QS21 adjuvants; AS07A contains CpG 7909. ⁵Inclusion required CR/Very Good Partial Response post-induction therapy.

Abbreviations: Ab, Antibody; ADCC, Antibody-Dependent Cellular Cytotoxicity; ASCI, Antigen-Specific Cancer Immunotherapeutic; CD4+, Cluster of Differentiation 4-positive; CD8+, Cluster of Differentiation 8-positive; Chemo, Chemotherapy; CpG ODN, Cytosine-phosphate-Guanosine Oligodeoxynucleotide; CR, Complete Response; dHER2, Recombinant HER2 Protein; dSLIM, Double-Stem-Loop Immunomodulator; HDIL-2, High-Dose Interleukin-2; IMO, Immunomodulatory Oligonucleotide; ISS, Immunostimulatory Sequence; MART-1, Melanoma Antigen Recognized by T-cells 1; ORR, Objective Response Rate; OS, Overall Survival; PD-1, Programmed Cell Death 1; PFS, Progression-Free Survival; PRAME, Preferentially Expressed Antigen in Melanoma; recMAGE-3, Recombinant Melanoma-Associated Antigen 3; RFS, Relapse-Free Survival; RT, Radiotherapy; SCT, Stem Cell Transplant; SOC, Standard of Care; TME, Tumor Microenvironment; TLR9, Toll-Like Receptor 9.

however, response rates vary across other melanoma trials.⁸⁵ Notably, the combination of CMP-001 and pembrolizumab achieved a 25% ORR (11/44 patients) in patients resistant to prior anti-PD-1 therapy, suggesting that TLR9 agonists may help overcome resistance to PD-1 blockade.⁸³ The promising, yet variable efficacy of Class A ODNs in melanoma likely reflects their reliance on type I IFN-mediated immune activation, a mechanism that is particularly effective in immunologically “cold” tumors with limited immune infiltration and intrinsic resistance to PD-1 blockade.⁸³ Consistent with this, CMP-001 appears to be more effective in noninflamed tumors.¹¹⁰ However, in tumors with highly immunosuppressive TMEs, including some melanoma subtypes, TLR9-driven antitumor immunity may be attenuated or suppressed. pDCs in these settings may be scarce, dysfunctional, or overwhelmed by competing immunosuppressive signaling, diminishing the type I IFN antitumor response.⁸³ This likely underlies the variable outcomes observed across trials. Overall, the Class A CpG agonists appear to depend on a TME that retains a functional pDC compartment lacking significant competing immunosuppression.

Class B CpG ODNs are characterized by a fully phosphorothioate backbone and incorporate one or more 6-mer CpG motifs.¹⁰⁸ CpG-7909 is the most extensively studied TLR9 agonist and has been investigated both as a monotherapy and in combination with various other cancer therapies, including chemotherapy, radiotherapy, peptide vaccines, and targeted therapies, across multiple tumor types (Tables 1 and 2). In pDCs, Class B ODNs are rapidly routed to lysosomes and therefore induce only weak type I IFN responses.^{6,109} Instead, they act predominantly on B cells to promote Th1-type immune responses and enhance MHC II expression on APCs.⁵ These mechanisms are particularly relevant in hematologic malignancies, which often contain a TME enriched with TLR9-expressing immune cells. Although Class B ODNs have demonstrated limited efficacy as monotherapy in solid tumors (Table 1), CpG-7909 achieved an objective response rate (ORR) of 32% (9/28 patients) in a Phase I study for CTCL, a disease driven by immature dendritic cells that promote Th2 polarized T cell proliferation and immune dysregulation.⁷⁶ In this setting, Class B TLR9 agonists act directly on malignant and infiltrating immune cells, enhancing antigen presentation and restoring a Th1-dominant antitumor response.⁷⁶ In solid tumors, however, Class B CpGs have demonstrated limited benefit even when used in combination regimens (Table 2). Non-small cell lung cancer (NSCLC) initially appeared to be a promising target for TLR9 modulators due to its immunosuppressive TME and expression of TLR9 in tumor cells and surrounding myeloid cells.⁸⁷ While preclinical studies and phase II trials suggested that CpG-7909-induced APC activation could synergize with cytotoxic taxane/platinum chemotherapy, this combination therapy failed to enhance clinical responses or extend progression free survival (PFS) in a Phase III NSCLC trial and was associated with significant adverse effects, including sepsis, despite improving objective responses in a phase II trial.^{87,88} These findings suggest that Class B ODN-driven pathways are insufficient to overcome the profound immunosuppressive TME typical of NSCLC. Additionally, systemic CpG agonist delivery may have primarily engaged the pro-tumorigenic NF- κ B/STAT3 axis of TLR9 signaling within NSCLC cells, effectively reinforcing the tumor’s growth pathways and negating any beneficial immune effects.^{38,41,87} It is possible that NSCLC may be better suited for treatment with Class A CpG ODNs, which induce robust type I IFN production and pDC activation that more directly counteract the myeloid suppression, defective antigen presentation, and impaired innate signaling characteristic of NSCLC. Similar patterns have been observed across other solid tumors. While Class B CpGs often elicit CD4+, CD8+, and/or humoral responses, these rarely translate into meaningful tumor regression.^{86,91,92}

In hematologic malignancies, Class B ODNs are most effective when paired with therapies that complement their immune-activating mechanisms. A phase II trial combining ISS-1018—a Class B CpG ODN primarily utilized as a vaccine adjuvant—with rituximab, reported an ORR of 48% (11/23) in patients with refractory follicular lymphoma.⁹⁴ In this context, Class B ODNs enhance rituximab-mediated antibody-dependent cellular cytotoxicity (ADCC) by triggering TLR9-dependent activation of APCs and subsequent cytokine-mediated activation NK cells, increasing antigen presentation and Fc-mediated cytotoxicity.⁹⁴ Class B ODNs can also synergize with radiotherapy which produces highly immunogenic tumor antigens, that, when coupled with TLR9-driven APC activation, enables APC cross-presentation and drives expansion of tumor-reactive CD8+ T cells.⁹⁰ In a phase I/II trial of low-grade B-cell lymphoma, the combined regimen of radiotherapy and CpG-7909 resulted in an ORR of 27% and increased tumor-reactive memory CD8+ T cells.⁹⁰ However, clinical responses were diminished in patients with Treg-inducing tumors, further reinforcing that the efficacy of Class B ODNs is contingent upon a permissive TME and suggesting that co-

administration with Treg-modulating agents like CTLA-4 inhibitors may be critical for success in immunosuppressive contexts.⁹⁰

Class C ODNs incorporate characteristics of both preceding classes and feature a fully phosphorothioate backbone and palindromic CpG motifs.¹⁰⁸ They elicit broader immune responses by inducing robust type I IFN responses while also promoting B cell, APC, and NK cell activation.¹⁰⁸ This may allow Class C ODNs to overcome the limitations of Class A and B ODNs. SD-101, a Class C ODN, has been combined with radiotherapy in lymphomas and pembrolizumab in metastatic melanoma, with promising early results (Table 2). In combination with pembrolizumab, SD-101 achieved clinical responses in 78% (7/9) of patients with PD-1 inhibitor-naïve tumors.⁸³ This exceeds the typical 35–40% clinical response rate typically observed with pembrolizumab monotherapy.⁸³ In this context, TLR9 agonism triggers robust innate activation that enhances T cell recruitment and priming, transforming the TME into a state that is conducive to checkpoint blockade.⁸³ However, it is unclear whether the moderate IFN- α induction of Class C ODNs will be sufficient to overcome the immunosuppressive TME of less immunogenic solid tumors. Several phase I/II trials are currently investigating SD-101 combination therapies in pancreatic (NCT04050085), prostate (NCT03007732), liver (NCT05220722), and breast (NCT01042379) cancers.

Modifications to the sequence length, shape, and delivery mechanism of TLR9 agonists have been pursued to potentially enhance their antitumor immune activity.¹⁰⁸ MGN1703, a TLR9 agonist with a double-stem loop structure, significantly improved PFS in metastatic CRC but did not improve clinical responses or PFS when combined with standard-of-care therapies in extensive-stage small-cell lung cancer (Tables 1 and 2). Immunomodulatory oligonucleotides (IMOs) contain CpR dinucleotides, which substitute the standard guanine nucleotide in CpG motifs with 7-deaza-guanosine to increase IFN- α secretion and B cell activation.¹⁰⁸ However, in clinical trials, neither IMO-2055 nor IMO-2125 improved clinical outcomes when combined with various targeted agents or immunotherapies. Additionally, one study evaluating IMO-2055 alongside 5-FU, cisplatin, and cetuximab (NCT01360827) was terminated due to serious adverse events (Table 2). These findings suggest that while modified TLR9 agonists may amplify innate immune signaling *in vitro*, this does not reliably translate into effective antitumor immunity in patients, potentially due to the constraints of an immunosuppressive TME. Collectively, the disappointing clinical performance of modified TLR9 agonists suggests that structural optimization alone is unlikely to improve therapeutic efficacy of TLR9 agonists, highlighting the need for combination strategies that directly address these immune constraints.

More recently, next-generation TLR9-modulating platforms have sought to overcome these limitations through precision delivery rather than sequence optimization. AST-008 (cavrotolimod) is a spherical nucleic acid-based TLR9 agonist, whose structure enhances cellular uptake and endosomal trafficking.¹⁰⁷ In combination with anti-PD-1 therapy, AST-008 led to an ORR of 11.7% (6/51) in patients with advanced skin cancers refractory to prior PD-1 blockade.¹⁰⁷ Similarly, Toll-like Receptor Agonist Antibody Conjugates (TRAACs), developed by Tallac Therapeutics, deliver highly potent CpG agonists directly to specific immune cell subsets. TAC-001, which conjugates a CpG agonist to an anti-CD22 antibody, selectively targets and activates CD22+ B cells to promote antitumor immunity while minimizing off-target systemic TLR9 activation.⁸¹ Early results from the ongoing phase I/II INCLINE-101 trial (NCT05399654) show that TAC-001 monotherapy achieved an ORR of 2.9% [2/69] in patients with advanced solid tumors refractory to immune checkpoint inhibitors.⁸¹ Another TRAAC, ALTA-002, targets SIRP α on myeloid cells and has demonstrated robust antitumor activity across multiple preclinical tumor models.¹¹¹ Collectively, these novel mechanisms of CpG delivery illustrate a shift towards targeted, cell-specific TLR9 activation, a strategy that may help overcome the intrinsic limitations of earlier TLR9 agonists while reducing systemic toxicity.

Tumor vaccines targeting cancer-associated peptides such as MAGE, PRAME, and NY-ESO-1 have incorporated TLR9 agonists as adjuvants to enhance antigen-specific immune responses. In this setting, TLR9 agonism provides a strong innate activation signal that supports the development of robust adaptive immune responses which are critical for effective vaccination.⁹⁵ The AS15 antigen-specific cancer immunotherapeutic (ASCI) adjuvant system—a liposomal formulation containing CpG-7909, 3D-MPL, and QS-21—induced durable antigen-specific CD4+ T cell and antibody responses in melanoma and multiple myeloma patients.^{95,96,98} However, most studies report limited expansion of cytotoxic CD8+ T cells, consistent with the signaling profile of Class B ODNs, which predominantly activate B cells and conventional APCs to generate a CD4+ Th1 response.^{95,96,98,99} These limitations suggest that Class A ODN which

trigger robust type I IFN signaling and pDC-mediated cross-priming, or Class C ODNs, which combine Class A and Class B mechanisms, may serve as more effective adjuvants when CD8⁺ T cell responses are required. Additionally, several studies highlight regulatory T cells as a barrier to vaccine efficacy, indicating that TLR9-based vaccine adjuvants may benefit from combination strategies that modulate Treg activity, such as immune checkpoint blockade.^{95,97,98}

Overall, TLR9 agonists show promise in cancer therapy due to their ability to activate both innate and adaptive immune responses, particularly when combined with treatments such as ICIs, chemotherapy, radiation, and tumor vaccines. It appears that the immunologic context in which TLR9 agonists are applied significantly influence their efficacy, potentially to a greater extent than the CpG chemistry itself. TLR9 signaling can generate potent innate and adaptive immune activation, but its antitumor effects depend on the presence of functional pDCs, effective antigen presentation, and a TME capable of supporting T cell recruitment and priming. Across studies, TLR9 agonists show the greatest activity when paired with therapies that relieve immunosuppressive pathways or provide complementary sources of tumor antigens. However, challenges remain due to safety concerns, variable clinical outcomes, and underwhelming results in phase III clinical trials. A clearer understanding of which TLR9-dependent pathways dominate in individual tumors and how each tumor's immune composition shapes those pathways will be important for refining the use of TLR9 agonists. As such, future progress will likely depend on developing more effective combination therapies and identifying biomarkers that can help guide patient selection and predict treatment response.

TLR9 Antagonists

Inhibiting TLR9 is a rational therapeutic approach in cancers where its activation drives tumor progression through chronic inflammation, immune evasion, and metastasis. In preclinical studies, chloroquine—a TLR9 inhibitor—reduces tumor size in mice with intrahepatic cholangiocarcinoma and suppresses proliferation and invasion in esophageal cancer models.^{3,112} Additionally, TLR9 antagonist ODN INH18 significantly reduces *in vitro* cervical cancer proliferation and induces apoptosis by decreasing Bcl-2 expression and increasing Bax.³⁷ Despite demonstrating some efficacy in preclinical models, TLR9 antagonists lack sufficient evidence to progress to clinical trials.

While most molecular studies highlight TLR9's role in tumor growth and progression, nearly all current treatment approaches use TLR9 agonists instead of antagonists. This discrepancy suggests that TLR9's ability to activate the immune system outweighs its tumor-promoting effects, especially when paired with therapies that reduce immunosuppression. Additional research is necessary to refine the design, delivery, and implementation of TLR9 modulators.

Role of TLR9 Beyond Cancer Infection

Upon bacterial or viral infection, TLR9 detects unmethylated CpG motifs in pathogenic DNA sequences and triggers a strong type I IFN response; however, TLR9 overactivation is observed in multiple viral infections.¹⁷ TLR9 contributes to symptom severity in COVID-19 patients and is central to immune dysregulation in HIV infection.^{113,114} Despite this, Class C CpG ODNs are effective at decreasing viral load and activating B cells, pDCs, and NK cells in chronic viral infections, such as hepatitis C.^{115,116} Additionally, as vaccine adjuvants, Class B CpG ODNs boost both humoral and cell-mediated immunity. Within two weeks, most recipients given CpG-7909 alongside the hepatitis B vaccine achieved protective anti-hepatitis B IgG levels, compared to none who received the vaccine alone.¹¹⁷ The TLR9 adjuvant group also developed a higher proportion of high-avidity antigen-specific antibodies.¹¹⁸ These improvements are particularly beneficial for immunocompromised patients, such as HIV⁺ individuals. When used as an adjuvant, CpG-7909 significantly boosted antibody titers, T cell responses, and long-term protection against hepatitis B in HIV⁺ recipients compared to the standard vaccine alone.¹¹⁹

Autoimmune Disease

In autoimmunity, TLR9 acts as both a promoter and suppressor of disease, depending on the context. In systemic lupus erythematosus (SLE), TLR9 directly recognizes self-DNA-containing immune complexes, leading to the release of IFN- α from pDCs and the activation of B cells, which perpetuates autoantibody production.¹²⁰ SLE patients exhibit increased

TLR9 expression, and targeted disruption of TLR9 signaling in B cells reduces inflammatory cytokines and alleviates symptoms.¹²¹ Conversely, TLR9-deficient lupus-prone mice exhibit increased pDC activation, IFN- α production, and disease severity despite reduced high-affinity autoantibodies.^{122,123} This suggests that TLR9's net effect is protective despite enhancing antibody affinity. In rheumatoid arthritis (RA) patients, TLR9 overexpression in monocytes and pDCs worsens symptoms, whereas TLR9 antagonism maintains remission in those at high risk for disease recurrence.^{124,125} TLR9 is also linked with the progression of both type I and type II diabetes; however, the exact mechanism is unclear and needs additional investigation.^{126,127}

Inflammatory Conditions

TLR9 plays a context-dependent role in inflammatory disorders such as inflammatory bowel disease (IBD) and allergies. In acute colitis, apical TLR9 suppresses gut inflammation via Treg activation, whereas in chronic IBD, high TLR9 expression correlates with disease severity and production of proinflammatory cytokines.^{128,129} Clinically, prophylactic CpG-ODN administration prevents intestinal inflammation in murine IBD, while ODN antagonists decrease gut inflammation and cytokine secretion.^{129,130} In clinical trials, DIMS0150, a TLR9 agonist, led to higher remission rates and increased mucosal healing in patients with ulcerative colitis.¹³¹ In allergies, including asthma and rhinitis, TLR9 ligands suppress inflammation and reprogram the allergic Th2-dominant immune response toward a Th1 profile.^{132,133} Using this approach, a TLR9-agonist-based vaccine containing a CpG-ODN conjugated to the ragweed allergen significantly improved symptoms in allergic rhinitis patients.¹³²

Conclusion and Future Directions

TLR9 exhibits multifaceted, context-dependent roles in humans, acting as a promoter of tumorigenesis, mediator of antitumor immunity, and regulator of immune responses in infection, autoimmunity, and inflammatory diseases. In this way, it is similar to other immunoregulatory agents—such as TLR3, inducible T-cell co-stimulator (ICOS), V-domain immunoglobulin suppressor of T cell activation (VISTA), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3)—that have both stimulatory and inhibitory effects in a context-dependent manner, and whose expression varies across cell types and between patients.^{134–140} While TLR9's immunomodulatory nature enables the recognition and targeting of pathogens and cancer cells, its overexpression in tumors contributes to chronic inflammation, cell survival, metastatic spread, and immune evasion. The pathways and consequences of TLR9 signaling in cancer vary by tumor type, stage, and microenvironment, with NF- κ B, STAT3, and COX-2 serving as key mediators. Despite this, therapeutic strategies to inhibit TLR9 are extremely limited, as TLR9 antagonists have shown minimal success in translational studies. The primary therapeutic interest in TLR9 centers on its ability to activate antitumor immunity, yet the immunologic context of the tumor greatly influences efficacy. Classic synthetic CpG ODNs activate pDCs, NK cells, and T cells, although their clinical efficacy is highly variable and frequently limited by an immunosuppressive TME. Consistent with these observations, Class A and C ODNs appear most effective in combination with ICIs, and Class B ODNs demonstrate potential alongside tumor vaccines. Importantly, differences in CpG ligand class, backbone chemistry, and structural configuration also determine whether TLR9 signaling is biased toward potent type I IFN production in pDCs or toward B cell, APC, and Th1-type activation, further contributing to heterogeneity of clinical responses observed in trials. Across cancer types, successful responses consistently correlate with TMEs that retain functional pDCs, intact antigen presentation, and the capacity to support type I IFN-driven immune priming. These insights have shifted TLR9 modulators away from simple sequence modification and toward strategies that more directly address TME constraints, including novel precision delivery technologies and synergistic immune combination regimens.

Future research should prioritize mapping TLR9-dominant signaling pathways within specific tumor contexts and identifying predictive biomarkers to stratify patients that are most likely to benefit from TLR9-based therapies. Given TLR9's dependence on intact pDC function, type I IFN signaling, and myeloid cells capable of supporting innate activation, biomarkers such as pDC abundance, IFN- transcriptional signatures, and NF- κ B/STAT3 expression patterns may help distinguish tumors with sensitivity versus resistance to TLR9 agonists. The development of circulating biomarkers—particularly cell-free DNA (cfDNA)-based assays—offers an additional opportunity to integrate TLR9 therapeutics with continuous tumor monitoring.^{141,142} Because cfDNA reflects tumor burden, mutational landscape, and

immune activity, pairing TLR9 agonists with cfDNA-guided diagnostics could enable adaptive treatment strategies, earlier identification of immune activation or resistance, and more precise patient selection.^{143,144} In parallel, recent advances in methylation-based liquid biopsy assays demonstrate how CpG modification patterns can be leveraged in clinical diagnostics. For example, the Belay Vantage™ assay uses enzymatic DNA conversion to evaluate *MGMT* promoter methylation in low input cerebrospinal fluid-derived cfDNA from patients with nervous system tumors, highlighting how CpG methylation profiling is increasingly being integrated into translational oncology workflows.¹⁴⁵ Because TLR9 activation is highly sensitive to CpG methylation status, these developments reinforce the potential for methylation-informed biomarkers to guide patient selection for TLR9-targeted therapies. Ultimately, the integration of biomarker profiling, cfDNA-guided monitoring, and optimized combination regimens has the potential to transform TLR9 modulators into more precise and reliable immunotherapeutic strategies.

Highlights

- TLR9 promotes and suppresses tumorigenesis via tumor- and context-specific pathways
- NF-κB/STAT3 signaling is central to TLR9-driven inflammation and tumor survival
- TLR9 activation in plasmacytoid dendritic cells and NK cells drives tumor apoptosis
- TLR9 agonist combinations activate antitumor responses in select patients/tumors
- Biomarkers are needed to stratify patients and predict responses to TLR9 modulators

Abbreviations

AP-1, Activator Protein 1; ADCC, Antibody-Dependent Cellular Cytotoxicity; APCs, Antigen-Presenting Cells; ASCI, Antigen-Specific Cancer Immunotherapeutic; Bax, Bcl-2 associated x protein; Bcl-xL, B-cell lymphoma-extra Large; CCL, C-C Motif Chemokine Ligand; CCR, C-C Motif Chemokine Receptor; CDK, Cyclin-Dependent Kinase; CLL, Chronic Lymphocytic Leukemia; COX-2, Cyclooxygenase-2; CpG, Cytosine-Phosphate-Guanosine; CRC, Colorectal Cancer; CTCL, Cutaneous T-Cell Lymphoma; CTLA-4, Cytotoxic T-Lymphocyte-Associated Protein 4; CXCL, C-X-C Motif Chemokine Ligand; CXCR, C-X-C Motif Chemokine Receptor; DAMPs, Damage-Associated Molecular Patterns; EBV, Epstein-Barr Virus; ECM, Extracellular Matrix; EMT, Epithelial-Mesenchymal Transition; ERK, Extracellular Signal-Regulated Kinase; EZH2, Enhancer of Zeste Homolog 2; GBM, Glioblastoma Multiforme; HCC, Hepatocellular Carcinoma; HIF-1, Hypoxia-Inducible Factor 1; HPV16, Human Papillomavirus 16; IBD, Inflammatory Bowel Disease; ICAM-1, Intercellular Adhesion Molecule 1; ICIs, Immune Checkpoint Inhibitors; ICOS, Inducible T-Cell Co-Stimulator; IFN, Interferon; IκB, Inhibitor of NF-κB; IL, Interleukin; IMOs, Immunomodulatory Oligonucleotides; IRAK, Interleukin-1 Receptor-Associated Kinase; IRF, Interferon Regulatory Factor; IRF-7, Interferon Regulatory Factor 7; MAL, MyD88 Adaptor-Like; MAPK, Mitogen-Activated Protein Kinase; Mcl-1, Myeloid Cell Leukemia 1; MDSCs, Myeloid-Derived Suppressor Cells; MHC II, Major Histocompatibility Complex Class II; MMP, Matrix Metalloproteinase; mtDNA, Mitochondrial DNA; MyD88, Myeloid Differentiation 88; NF-κB, Nuclear Factor-Kappa B; NK, Natural Killer; NSCLC, Non-Small Cell Lung Cancer; ODNs, Oligodeoxynucleotides; OSCC, Oral Squamous Cell Carcinoma; PAMPs, Pathogen-Associated Molecular Patterns; PARP, Poly(ADP-Ribose) Polymerase; PD-1, Programmed Cell Death Protein 1; PD-L1, Programmed Death-Ligand 1; pDCs, Plasmacytoid Dendritic Cells; PFS, Progression-Free Survival; PMN-MDSCs, Polymorphonuclear Myeloid-Derived Suppressor Cells; PRR, Pattern Recognition Receptor; RA, Rheumatoid Arthritis; RCC, Renal Cell Carcinoma; ROS, Reactive Oxygen Species; SCIMP, Src Kinase-Interacting Membrane Protein; SLE, Systemic Lupus Erythematosus; SNAIL, Snail Family Transcriptional Repressor 1; STAT3, Signal Transducer and Activator of Transcription 3; TAMs, Tumor-Associated Macrophages; Th1, T Helper 1; TIM-3, T Cell Immunoglobulin and Mucin Domain-Containing Protein 3; TIMP, Tissue Inhibitor of Metalloproteinase; TLR9, Toll-Like Receptor 9; TME, Tumor Microenvironment; TNF, Tumor Necrosis Factor; TRAAC, Toll-Like Receptor Agonist Antibody Conjugate; TRAF6, TNF Receptor-Associated Factor 6; TRAIL, TNF-Related Apoptosis-Inducing Ligand; Tregs, Regulatory T Cells; TRIF, TIR-domain-containing adaptor protein-inducing beta interferon; TWIST1, Twist Family bHLH Transcription Factor 1; VEGF, Vascular Endothelial Growth Factor; VISTA, V-domain Immunoglobulin Suppressor of T Cell Activation; ZEB2, Zinc Finger E-box-Binding Homeobox 2.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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