Multiple Factors Determine the Oncolytic or Carcinogenic Effects of TLRs Activation in Cancer

Yingxiang Yang, Chengyue Jin, Anthony Yeo, and Bo Jin

1Department of Hepato-Pancreato-Biliary Surgery, The Sixth Medical Center of PLA General Hospital, Beijing 100048, China
2Beijing Arion Cancer Center, Beijing 100070, China
3NSW, Australia
4Senior Department of Gastroenterology, The First Medical Center of PLA General Hospital, Beijing 100853, China

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Toll-like receptors (TLRs) belong to a germline-encoded protein family. These are pattern recognition receptors. They sense pathogen-associated molecular patterns (PAMPs). When this occurs, activation of the NF-κB pathway follows. This triggers the innate immune response of the host. The consequent inflammatory cytokine response usually contributes to the elimination of the pathogen. Activation of TLRs also induces an adaptive immune response by a cross-prime mechanism. This mechanism is employed in cancer immunotherapy. Using TLR ligands as adjuvants induces upregulation of costimulatory signals which in turn activates a cytotoxic leukocyte response against cancer cells. However, TLRs are also overexpressed in human cancer cells resulting in increased cell proliferation, migration, invasion, and angiogenesis. An intracellular adaptor, myeloid differentiation factor 88 (MyD88) probably mediates this process. MyD88 is intimately involved with all TLRs except TLR3. One consequence of the interaction between a TLR and MyD88 is activation of NF-κB. In this context of a variety of proinflammatory cytokines being produced, chronic inflammation may result. Inflammation is an important protective mechanism. However, chronic inflammation is also involved in carcinogenesis. Activation of NF-κB inhibits apoptosis and under certain circumstances, tumor cell survival. In this review, the potential therapeutic value of TLRs in immunotherapy and its role in oncogenesis are explored. The emerging use of artificial intelligence is mentioned.

1. Introduction

Toll protein was initially discovered in Drosophila. In humans, a homologue protein of Drosophila Toll was discovered in 1997 and is referred to as toll-like receptor 4 (TLR4). TLR4 activates the NF-κB pathway to initiate the production of inflammatory cytokines [1]. Similar proteins that participate in this response are referred to as toll-like receptors (TLRs) [2].

TLRs are germline-encoded pattern recognition receptors (PRRs) that play a central role in host cell recognition and response to microbial pathogens. TLR-mediated recognition of microbial products from various pathogens serves as signatures for each microbial species. These microbial products are termed pathogen-associated molecular patterns (PAMPs). TLRs also recognize damage-associated molecular patterns (DAMPs) released from dying cells. Innate immune cells can sense PAMPs of the invading microbe or DAMPs from the dying host cell to produce proinflammatory cytokines. Additionally, activation of TLRs can occur in professional antigen-presenting cells (APCs) such as dendritic cells (DCs). DCs activate T lymphocytes. This is the adaptive immune response. As TLR agonists trigger innate immunity and shape adaptive immunity, they have been investigated in cancer clinical trials [3]. Three TLR agonists have been approved for clinical use by the US Food and Drug Administration (FDA) [3].

TLRs have been implicated in many chronic diseases, e.g., inflammatory bowel diseases, psoriasis, inflammation of the central nervous system, diabetes mellitus, systemic lupus erythematosus, and rheumatoid arthritis [4]. Disease chronicity is mediated by the secretion of growth and survival factors, proangiogenic factors, extracellular matrix modifying enzymes,
and reactive oxygen species [5]. An anticancer immune response or conversely, tumorigenesis can result.

2. TLRs and Cancer

TLRs are a group of transmembrane proteins sharing a common domain pattern. Every living eukaryotic organism has a different number of TLRs. In humans and mice, there are 10 TLRs and 12 TLRs, respectively [6]. Human-encoded TLRs are classed as TLR1–TLR10. Mice encoded TLRs are classified as being TLR1–TLR9 and TLR11–TLR13 [7, 8].

TLRs in human and mice genomes can be functionally divided into two subgroups based on their location in the cell and their ligand types. The first subgroup in humans consists of TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 and in mice, TLR1, TLR2, TLR4, TLR5, TLR6, TLR11, and TLR12. This subgroup is located on the cell membrane. The other subgroup in humans consists of TLR3, TLR7, TLR8, and TLR9. In mice, it is TLR3, TLR7, TLR8, TLR9, and TLR13. This subgroup is located in endosomes.

TLRs are synthesized in the endoplasmic reticulum (ER) and transported to the cell membrane or endosome. The endosomal TLRs reside in ER in nonstimulated cells. The intracellular transportation of cell surface TLRs occurs via the traditional route to the Golgi complex. When the cells are stimulated by ligands of endosomal TLRs, these TLRs will be transported to the endolysosome.

The endosomal TLRs are proteolytically cleaved at their ectodomain by cathepsins in the endosome. This cleavage is required to form functional dimerized TLRs. Although the uncleaved endosomal TLRs are capable of binding to their ligands, such bindings do not trigger signal transduction [6]. In contrast, transportation of cell surface TLRs occurs via the conventional secretory pathway, i.e., being transported from the ER to the Golgi apparatus and subsequently to the plasma membrane via secretory vesicles [6].

The different locations of TLRs may explain their different functions. The cell surface TLRs mainly sense the surface molecules of the invading microbes, e.g., lipids, lipoproteins, or proteins. Endosomal TLRs mainly sense the invading viral nucleic acids which are protected by viral capsid from degradation by extracellular and cytoplasmic nucleases.

2.2. TLRs Signal Pathways. Each member of the TLR family senses a distinct constituent of the invading microorganism (Table 1). This may ensure that the invading pathogen is recognized efficiently. Additionally, it may activate different TLRs to produce inflammatory cytokines against the pathogen.

A key point of the signaling pathway occurs when a ligand is recognized and two TLR molecules dimerize to form a homodimer or a heterodimer. With the exception of TLR3, all TLRs use Toll/IL-1 receptor (TIR) domain-containing adapter protein (TIRAP)/myeloid differentiation factor 88-adapter-like (Mal), to bind the adaptor protein myeloid differentiation factor 88 (MyD88) for downstream signaling. In contrast, TLR3 uses TIR domain-containing adapter-inducing interferon-β (TRIF) as its sole adaptor and TLR4 uses TRIF as an adjunctive adaptor. With the exception of TLR3, all dimerized TLRs combine TIRAP/Mal by associating the TIR domains of TLR with the TIR domains at the C-terminus of TIRAP/Mal. A complex is formed. This complex then combines with the adaptor protein MyD88.

MyD88 uses its N-terminal death domain (DD) to bind to the same domain of IL-1R-associated kinase-4 (IRAK4) [21]. IRAK4 then binds and phosphorylates IRAK1 and IRAK2. A higher order signaling complex, myddosome is formed and is responsible for downstream signaling [22]. The schema of the cell surface TLRs signaling pathway is shown in Figure 1. TRIF is the only adaptor of TLR3. TLR4 can use both TRIF and MyD88 as adaptors. Upon sensing a dsRNA ligand, the TLR3 TIR domain associates TRIF TIR directly. The association of TLR4 and TRIF is mediated by TRIF-related adaptor molecule (TRAM). The endosomal TLRs signaling is shown in Figure 2.

2.3. TLRs Activation in Immune Response and Inflammation. TLRs trigger signal cascades to induce a proinflammatory immune response to clear an infection [23]. Bacterial, viral, or fungal components, cytokines or TLR agonists stimulate, and educate cells involved in innate immunity. Thereafter,
metabolic and epigenetic reprogramming occurs in the educated innate immune cells. When these educated cells encounter a second stimulus, they exhibit increased or decreased cytokines or chemokines production. This trained innate immunity may persist for years [6].

Trained immunity can provide cross-protection. The mechanism of trained immunity may partially be due to TLR-induced chromatin modifications. Upon repeated stimulation of TLRs, such chromatin modifications induce two effects: protecting the host from infection and preventing excessive inflammation associated with infection [24].

DCs maturation is a prerequisite for the induction of the adaptive immune response. TLRs are involved in DCs maturation to induce the expression of signal 1 through signal 3 [25]. Antigens that are captured by DCs are capable of activating distinct TLRs. This results in DCs maturation. The mature DCs load the processed antigen epitopes onto class-II major histocompatibility complex (MHC-II) molecules and
present them along with costimulatory molecules to effector T cells. In this manner, T cells are informed of the characteristics of the invading pathogen. It also instructs T cells to differentiate into the appropriate type of effector cells by releasing different cytokines from mature DCs. Mature DCs induced by TLRs activation can also load antigen epitope to class I MHC molecules and present them to CD8$^+$ T cells. A cytotoxic immune response materializes. This process is called cross-presentation. TLR signals also influence the B cell response. B-1a cells can be rapidly recruited to sites of infection and produce antibodies independent of T cells. These cells are the main sources of serum natural IgM, microbiota reactive, and class-switched IgG and IgA antibodies. Both B cell receptors and TLRs generate nuclear factor-κB (NF-κB) signaling and natural immunoglobulin IgM production. Antibody production of a stable B-1a response to both self and gut microbial antigens requires both B cell receptors and TLRs activation [26].

Innate immunity is the major mechanism of inflammation and inflammatory diseases. Upon recognition of PAMPs, TLRs in innate cells are activated. However, under certain conditions, this response can become exaggerated or uncontrolled. A severe systemic inflammation or chronic inflammation may develop
Besides the PAMPs from invading microbes, TLRs can also recognize endogenous ligands from the host. These are nucleic acids, intracellular proteins, oxidatively modified lipids, extracellular matrix components, and other soluble mediators that are released by damaged tissues and dying cells. Endogenous ligands belong to the DAMPs family. Such engagement of TLRs with endogenous ligands induces the activation of the TLRs downstream signaling pathways (see Figure 3). Activation promotes the production of proinflammatory cytokines, chemokines, and interferons (IFNs). Indeed, the pathogenesis of inflammatory conditions such as rheumatic diseases, cancer, and wound healing involves endogenous ligands [27]. However,
It has been argued that some endogenous ligands of TLR2 and TLR4 may act as PAMP-binding molecules or PAMP-sensitizing molecules. These enhance the sensitivity of compromised tissues to potential microbial challenges. This argument is based on the possibility that lipopolysaccharides (LPS) and lipopeptides from experimental systems may be present and thus no definitive conclusions can be made [28].

2.4. TLRs-Induced Cellular Immunity Is Correlated with Cancer Rejection. When cells undergo an epithelial-to-mesenchymal transition (EMT) process, some transformed cells can express antigens that are not expressed or are found only in trace amounts in healthy hosts. These are known as tumor-associated antigens (TAAs). In this context, host immunity is usually weak or anergic due to either the weak antigenicity of TAAs or due to immune suppression. Activation of TLRs is capable of activating APCs which results in increased capacity of antigen processing. APCs then present the processed TAA epitopes to CD4⁺ or CD8⁺ T lymphocytes to induce type 1 T helper cell (Th1)-biased and/or cytotoxic immune responses. Such immune responses reject the transformed cancer cell [29]. This has been tested in a clinical trial using a TLR3 agonist in combination with local radiotherapy. Killed tumor cells release TAAs. These then cross-primed DCs. Subsequently, significant tumor rejection occurred. An increase in positive therapeutic outcomes was achieved when programed death protein-1 (PD-1) blockade was added to this treatment regimen [30]. A number of clinical trials using TLR agonists/antagonists in attempting to cure cancer are in progress (see Appendix Table S1).

The cancer milieu is called the tumor microenvironment (TME). TME is a unique milieu and is composed of numerous components, cancer cells, epithelial cells, fibroblasts, immune cells, and noncellular components such as proteins from the extracellular matrix. In progressive cancer, TME also mediates immune evasion and this may involve resistance to therapy [31]. The key factor correlated with the immune resistance lies with the antigen-presentation capacity of the tumor-associated macrophages (TAMs). TAMs in the immunosuppressive tumor are inactive and not able to present antigen. Use of TLR agonists such as TLR9 agonist unmethylated deoxycytosine preceding deoxyguanosine (CpG) oligoDNA or TLR3 agonist polyinosinic-polycytidylic acid (poly I:C) as adjuvants and pairing it with a nanogel based antigen produces TAMs. These TAMs have antigen-presentation activity. TAMs convert a tumor from being immunoresistant to being immunosusceptible to T lymphocytes therapy [32]. In a murine model where colon carcinoma MC38 cells are transplanted, host M2 phenotype macrophages can be converted into the M1 phenotype. TLR3 ligand poly I:C plays a critical role in this transformation. These converted macrophages exhibit enhanced antigen uptake and T cell activation. Consequently, tumor regression in a IFN-α/β signaling-dependent manner can occur [33]. TAMs are also associated with chemotherapy resistance. Application of R848, a TLR7/8 agonist can drive the differentiation of myeloid-derived suppressor cells (MDSCs) toward a tumoricidal M1 phenotype. It can also reverse chemoresistance to oxaliplatin in colorectal cancer [34]. Most studies have suggested that application of TLR agonists causes MDSCs to have a dominant immunosuppressive M2 phenotype which causes tumor rejection. However, other studies suggest that TLRs activation induces immunosuppressive M2 phenotype macrophages. This different response may be due to the type of TLR involved. The dual roles of TLRs stimulation on MDSCs have been reviewed elsewhere [35].

PD-1 is an inhibitory receptor protein expressed in immune cells especially in tumor-associated T lymphocytes. It binds to programed death ligand 1 (PD-L1) and programed death ligand 2 (PD-L2). These regulate T lymphocyte activation. When T cell receptor (TCR) binding occurs coincidently with PD-1 binding in T lymphocytes, PD-1 signaling inhibits phosphorylation of key TCR signaling intermediates. Hence, TCR signaling is terminated. With termination, there is suppression of T lymphocyte activation. Cancer cells that can express high levels of PD-L1 capable of binding to PD-1 in T cells have a negative effect on cytotoxic T lymphocytes [36]. Application of monoclonal antibodies to block PD-1 or PD-L1 has demonstrated promising therapeutic efficacy in various types of cancer. Immune checkpoint inhibitor therapy with antibodies against PD-1 or PD-L1 may achieve 20%–30% remission of solid tumors. Immunotherapy combined with radiotherapy or with chemotherapy or with a biological therapy likely improves the efficacy of PD-1/PD-L1 antibodies [37]. In the murine model with noninflamed lung tumor TC-1/A9, mice were treated with all possible combinations of a human papillomavirus E7 long peptide (a constitutively expressed tumor-associated peptide), poly I:C and anti-PD-1 monoclonal antibody. The maximum antitumor efficacy was achieved with intratumoral administration of HPV E7 long peptide, poly I:C, and systemic administration of anti-PD-1 antibody [38]. In humans, activation of TLR3 by poly I:C upregulates MHC-I and PD-L1 expressions in neuroblastoma cells. Considering both mouse and human data, it is hypothesized that increased PD-L1 levels caused by the combination of PD-L1 antibody and poly I:C may maintain the T cell stimulatory effects of MHC-I upregulation and suppress immune inhibition [39].

2.5. TLRs Are Involved in Carcinogenesis. The thesis of a linkage between chronic inflammation and cancer was proposed more than 160 years ago [40]. Chronic inflammation has been implicated in breast, liver, bowel, urinary bladder, prostate, gastric mucosa, ovarian, and skin cancers [41]. Globally, an estimated 15% of human cancers are related to inflammation [42].

2.5.1. TLR2. In gastric cancer patients, overexpression of TLR2 with corresponding mRNA predicted a higher degree of histological neoplasia and poor prognosis [43]. Activation of TLR2 induced tumor cell proliferation, enhanced generation of reactive oxygen species, and oxidative phosphorylation [43]. Stimulation of TLR2/TLR1 with its ligand, PamiCSK4 induces proliferation of lung adenocarcinoma cells in an NF-κB signaling-dependent manner [44]. Excessive TLR2 expression has also been found in oral squamous cell carcinoma, pancreatic intraepithelial neoplasia, and intrahepatic cholangiocarcinoma...
[45]. Silencing TLR2 reduced the expression of EMT markers, cancer cell migration, and invasion [46]. Inhibition of TLR2 or its downstream adaptor molecules MyD88 and IRAK1 inhibits human breast cancer proliferation. In colitis induced by dextran sodium sulfate, deletion of MyD88 or TLR2 in the intestinal epithelium reduced regeneration. It was also associated with reduced spontaneous development of colonic cancer [47]. Similar results have been found in mice gastric cancer [48].

2.5.3. TLR4. TLR4 uses both MyD88 and TRIF as its dual adaptors. Endogenous and exogenous LPS are the main ligands for TLR4. In addition, it can be engaged by some internal DAMPs which can be produced during the development of cancer [55]. Long pentraxin 3 is an acute-phase inflammatory glycoprotein produced by invasive melanoma cells. This glycoprotein can induce cellular migration, invasion, and the expression of EMT factor TWIST1 through a TLR4-dependent pathway [56]. Colonic epithelial cells that have been signaled by TLR4 expressed dual oxidase 2 and H_2O_2. Increased production of H_2O_2 is associated with colitis-associated dysplasia and carcinogenesis. TLR4-shaped microbiota is critical for TLR4-dependent colonic carcinogenesis. Transplantation of TLR4-shaped microbiota to wild-type mice can result in transferring epithelial redox activity and enhanced carcinogenesis [57]. Blocking TLR4 signaling by a specific inhibitor of TLR4, TAK-242, can reduce the invasive- ness of ovarian and breast cancer cell lines. This effect is mediated by two factors: decreased enzymatic activity of matrix metalloproteinase (MMP) 2 and MMP9, and down-regulated gene expressions of EMT-related genes [58]. Arresting TLR4 signal also increases the sensitivity of breast and ovarian cancer cells to chemotherapeutic drugs [59].

2.5.4. TLR5. A single dominant nucleotide polymorphism of TLR5, TLR5 R392X (1174C>T) is present in about 7.5% of the general population. This polymorphism results in a truncated transmembrane domain of TLR5. This affects the TLR5 response. It reduces signaling by 50%–80%. A survival analysis of two groups was conducted in sequenced samples of estrogen receptor-positive breast cancer patients from the Cancer Genome Atlas datasets. One group consisted of patients with TLR5 R392X who did not respond to bacterial flagellin. The other group consisted of patients without the nucleotide polymorphism TLR5 R392X. Nonresponders to bacterial flagellin had accelerated cancer progression. In contrast, TLR5-defective patients with ovarian cancer had a higher proportion of long-term survivors. An association with increased levels of IL-17 in TME was present. Decreased serum IL-6 levels were found in ovarian cancer patients with TLR5 R392X. However, in breast cancer patients with TLR5 R392X, there were no significant changes in serum IL-6 levels [60].

Experiments using TLR5-responsive mice demonstrated that commensal bacteria activated TLR5 and induced IL-6 production resulting in MDCs recruitment and activation of γδ T cells. Such cells then produced galectin-1. Impairment of anticancer immunity then occurred with predicted cancer progression. In TLR5 nonresponsive mice, IL-17 production is increased and promotes IL-6-unresponsive tumor progression. Elimination of commensal bacteria by antibiotics abrogates differences in tumor progression in both TLR5-responsive and nonresponsive mice [60]. In humans, TLR5 is activated by flagellin in gastric cancer cells which results in IL-8 production and NF-κB activation signaling. Cell proliferation then occurs [61]. TLR5 activation which promotes cancer progression. These have been found in skin, salivary gland, colorectal, and cervical neoplasia [62].

2.5.5. TLR7. TLR7 is expressed in human pancreatic cancer cells and surrounding stroma cells in a highly inflammatory niche. Activation of TLR7 in a murine pancreatic cancer model significantly promotes tumor progression via changes
in the gene expression involved in the regulation of growth. Inhibition of TLR7 protects mice from pancreatic cancer development. TLR7 signaling in inflammatory cells present in the pancreas is critical for pancreatic cancer progression [63]. The gene expression of TLR7 is significantly increased in the early stages and even more so in advanced stages of pancreatic cancer. Thus, a stage-dependent upregulation pattern of TLR7 expression is possible [64].

TLR7 is also expressed in human and murine lung cancer cells. In humans, stimulation of TLR7 with its agonist loxoribine causes NF-κB activation and increased expression of the antiapoptotic protein Bcl-2. It also prolongs survival of cancer cells and induces chemoresistance [65]. In the murine model, loxoribine injected into animals with implanted TLR7-positive human lung adenocarcinoma cells, increased tumor progression was observed. Chemotherapeutic resistance can also occur, e.g., this was observed in patients who underwent surgery for non-small cell lung cancer (NSCLC) that have high TLR7 levels. Poor clinical outcomes were seen due to resistance to neoadjuvant chemotherapy [66].

In human hepatocellular cancer (HCC) cells, TLR7 expression is upregulated. Stimulation of TLR7 significantly increases HepG2 cell proliferation and migration. Blockade of TLR7 signaling by a TLR7 antagonist 20S-protopanaxadiol significantly reduced HepG2 cell migration [67]. However, when a TLR7 agonist imiquimod is used on human ovarian cancer cell lines CaOV3 and SKOV3, an upregulation of mesenchymal phenotypes occurred in SKOV3 cells. It also induced EMT-related cytokines production in SKOV3 cells. This did not occur in TLR7 expressing CaOV3 cells [68]. Therefore, the effects of TLR7 activation in cancer cells may be a function of the cell type.

2.5.6. TLR8. Some reports suggest that TLR8 activation may be involved in oncogenesis and metastasis. MicroRNAs miR-21 and miR-29a secreted by cancer cells can be taken up by immune cells. These microRNAs are able to reach the endosomes and engage TLR8 to induce a prometastatic inflammatory response which may ultimately be oncogenic [69]. In an in vitro prostate cancer model, lactate released by glycolytic cancer-associated fibroblasts causes cellular EMT engagement. There was also upregulation of TLR8 and miR-21 which caused NF-κB-mediated inflammatory signaling. This promotes EMT induction and cellular invasion [70].

Stimulation of TLR8 in human lung cancer cells leads to atypical NF-κB activation. It also promotes cancer cell survival and chemoresistance [65]. Similar results were reported in pancreatic cancer where TLR7 and TLR8 expression were increased. Late stage cancers have increased amounts of TLR7 and TLR8. Resiquimod (R848) is an agonist for murine TLR7 and for human TLR7/TLR8 [71]. In a pancreatic cancer cell line (PANC1) when TLR7 and TLR8 were activated with resiquimod (R848), NF-κB and cyclooxygenase-2 expression increased. This resulted in cancer cell proliferation and resistance to chemotherapeutic agents. In Balb/c nude mice, resiquimod treatment induced TLR7 and TLR8 which promoted the growth of a xenograft consisting of human PANC1 cancer cells [64]. In patients with colorectal cancer, TLR8 expression was found to be a prognostic factor. Higher levels of TLR8 predict a worse clinical outcome. Expressions of TLR7 and TLR8 were associated with CD133+ colorectal cancer stem cells. Sustained activation of NF-κB signaling promotes colorectal cancer growth and progression with enhanced ability to resist apoptosis [72]. However, in patients with HCC, TLR8 expression was not associated with survival [73].

2.5.7. TLR9. TLR9 is an endosomal TLR sensing DNA with a CpG motif. It is expressed in a variety of cancer cells [74]. When cancer cells are killed by chemotherapy, the DNA released is acted by cell death-activated DNAases to form specific structures. These specific DNA structures may be nucleosome resistant, e.g., telomeric G-quadruplex-forming DNA fragments [75]. Such DNA fragments can be taken up by cancer cells in a LL-37 regulation pattern. LL-37 is a human antimicrobial cathelicidin peptide present in breast cancer. It is involved in the entry of DNA fragments into cancer cells. Within the cells, such DNA fragments activate TLR9. The activation of TLR9 induces cellular invasion mediated by cathepsins and MMPs. Compared to treatment with DNA fragments alone, LL-37 bound to DNA fragments increased the cellular uptake of these DNA fragments. However, cancer cell invasiveness was decreased in a dose-dependent manner [75].

Cellular mitochondrial fission causes mitochondrial DNA (mtDNA) leak into the cytosol. These mtDNA molecules activate the TLR9-mediated NF-κB signaling pathway to promote CCL2 secretion in HCC cells. This induces TAMs recruitment and polarization toward the M2 phenotype, promoting HCC progression [76]. The hypoxia likely present in the center of a large tumor can result in cell death. Formation of DAMP molecules such as high mobility group box 1 (HMGB1) and mtDNA then results. A hypoxic environment can promote HMGB1 translocation from the nucleus to the cytosol and interact with mtDNA released from the damaged mitochondria. Consequently, TLR9 activation occurs. HCC cell proliferation concomitantly occurs [77]. Activation of TLR9 in HCC cells also increases PD-L1 expression to suppress an anticancer immune response. Suppression of antitumor immunity resulted when TLR9 activation upregulated PD-L1 expression in HCC cells. This may suggest that combining a TLR9 agonist and an anti-PD-1 antibody is a distinct antitumor therapeutic possibility. Immune checkpoint blockade can overcome the immunosuppression induced by TLR9 activation, while TLR9 agonists can enhance anti-PD-1 therapy response rates [78]. TLR9 expression was also found to be associated with PD-L1 expression in peripheral T cell lymphoma. Elevated TLR9 and PD-L1 expressions were unfavorable prognostic factors for patients with peripheral T cell lymphoma [79]. In prostate cancer, high expression of TLR9 was associated with an increased likelihood of lymph node metastasis and poor prognosis. Silencing of TLR9 by siRNA inhibited prostate cancer cell line, PC-3 cells migration, and invasion [80]. Similar results were reported with esophageal cancer and glioma [81].

2.6. TLR-Induced Cytokines Are Involved in Carcinogenesis. Proinflammatory cytokines are usually downstream products of TLR activation signaling. Both PAMPs from invading
pathogens or DAMPs from endogenous cells can activate TLR signaling and promote proinflammatory cytokines. These cytokines are involved in carcinogenesis (Figure 3).

Tumor necrosis factor (TNF) is among one of the earliest discovered proinflammatory cytokines. Macrophages in TME produce TNF and IL-6 to trigger NF-κB and signal transducer and activator of transcription 3 (STAT3) signaling in gastric cancer cells. This culminates in PD-L1 production. The result is a proliferation of cancer cells due to inhibition of T cell cytotoxicity [82]. Animal experiments have demonstrated that knockout of TNF-α or its receptor TNFR1 significantly suppressed gastric cancer cell proliferation in mice [83]. In a colitis-related colonic cancer model, blocking TNF-α with etanercept, a specific antagonist of TNF-α, significantly suppressed the onset of colonic cancer in mice [84].

IL-1 consists of IL-1α and IL-1β, binding to a complex receptor composed of interleukin-1 receptor 1 (IL-1R1) and interleukin-1 receptor accessory protein (IL-1RACp). IL-1α is expressed in a variety of tissue cells and is transformed into p17 by proteolysis and released when cellular death occurs. IL-1β is expressed in a variety of tissue cells and is transformed into interleukin-1 receptor accessory protein (IL-1RAcP). IL-1 receptor composed of interleukin-1 receptor 1 (IL-1R1) and IL-1RAcP. IL-1α is more proinflammatory than IL-1α. IL-1β is regarded as a local inflammatory cytokine. IL-1β is expressed in myeloid cells and is involved in a local and systemic inflammation [85]. It is increased in human and mice pancreatic ductal adenocarcinoma cells through TLR4/nucleotide oligomerization domain (NOD)-like receptor (NLR) pyrin domain 3 (NLRP3) inflammasome signaling. Cancer cell-produced IL-1β can induce accumulation of immunosuppressive cells, such as M2-type macrophages, tumor-associated neutrophils, Th17 cells, MDSCs, and regulatory B cells. This creates an immunosuppressive TME in the mice model of pancreatic ductal adenocarcinoma [86]. An in vitro study demonstrated that both IL-1α and IL-1β induce bladder cancer cell migration and invasion with IL-1/β playing a more potent role than IL-1α [87].

IL-6 is a major inflammatory cytokine. The IL-6 family contains several members, including IL-6, IL-11, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, cardiotrophin 1, cardiotrophin-like cytokine, and IL-27. They share a common subunit, glycoprotein 130 (gp130) in their receptor complex [88]. IL-6 can induce chemoresistance and influence cancer cell survival. It also promotes cancer cell migration, invasion, and angiogenesis [89]. Accumulation of IL-6 in some organs such as the brain, lung, liver, or bone marrow may attract circulating tumor cells to these organs and promote their residence and progression into metastatic lesions [90]. It also plays an important role in the generation and maintenance of some cancer stem cells [89]. Activation of epidermal growth factor receptor signaling and oncogenic K-ras mutation produces IL-6 [91]. Increased levels of IL-6 induce tumorigenesis with cancer cell resistance to ferroptosis [92]. It is also involved in the communication between cancer cell and cancer-associated fibroblast in the TME in a paracrine manner. Suppression of IL-6 signaling can be achieved by tocilizumab, an antibody to IL-6 receptor α. Tumor growth is correspondingly reduced [93].

IL-11 belongs to the IL-6 family. Experiments in the mice gastric cancer model demonstrated that IL-11 is capable of activating transcription factor STAT3 to induce overexpression of absent in melanoma 2 (AIM2). AIM2 is a PRR in cytosol sensing DNA from invading pathogen. AIM2 then interacts with microtubule-associated end-binding protein 1 to enhance tumorigenesis and cancer cell migration. Clinically, overexpression of AIM2 and microtubule-associated end-binding protein 1 is associated with poor prognosis in gastric cancer patients [94]. Similar results were observed in NSCLC. In addition, IL-11 can induce an EMT process [95]. IL-11 is more potent than IL-6 in STAT3 activation and carcinogenesis. Suppression of IL-11 signaling can downregulate STAT3 activation, tumor cell proliferation, and inhibit cancer cell invasion [96]. Suppressor of cytokine signaling 3 (SOCS3) is a negative regulator of gp130 signaling. Mice with the genetic modification of gp130Y757F/Y757F are unable to combine SOCS3 resulting in enhanced STAT3 signaling. These mice tend to spontaneously develop gastric hyperplasia and cancer. Increased expression of IL-11 was observed in gp130Y757F/Y757F mice that have gastric cancer. However, when such gp130Y757F/Y757F mice lacked IL-11 receptor, levels of IL-11 were normal. Gastric cancer did not occur. Suppression of IL-11 downstream transcription factor STAT3 and STAT1 in gp130Y757F/Y757F mice reduced gastric IL-11 expression and decreased cancer development [97].

IL-8 is another cytokine regulated by the NF-κB signaling pathway. The levels of serum IL-8 have a high correlation with tumor burden and metastasis in melanoma and NSCLC patients [98]. It is a prognostic indicator of poor survival [99]. Higher levels predict a worse outcome. IL-8 is capable of inducing angiogenesis and recruiting neutrophils to the tumor site in a paracrine manner. It can induce oncogenic signaling, prometastatic invasion, and chemoresistance. IL-8 expression also induces MDSCs. This creates an immunosuppressive microenvironment [99]. Endothelial cells can be stimulated by IL-8 to produce MMPs capable of degrading the surrounding extracellular matrix [100]. IL-8 is involved in the promotion of EMT processes [101], the development of cancer stem cells [102], and to initiate cancer cell growth and invasion. It is also capable of activating cancer-associated adipocytes with consequent malignant growth and invasion [103].

IL-10 is a major anti-inflammatory cytokine and negatively regulates the immune responses. It can be produced by both myeloid and lymphoid cells including CD4+ and CD8+ T cells, B cells, macrophages, monocytes, DCs, neutrophils, mast cells, eosinophils, and NK cells [104]. The role of IL-10 appears paradoxical: tumor promoting and tumor suppressive. IL-10 is able to inhibit antigen presentation and proinflammatory cytokines production. A number of clinical studies have reported that increased levels of IL-10 in serum or cancer tissues were correlated with poor prognosis [105]. For example, melanoma patients with serum IL-10 level > 10 pg/ml had poorer survival compared to those with a level <10 pg/ml (365 vs. 557 days, respectively, p = 0.0259) [106]. However, the presence of IL-10 can suppress the simultaneous production of other proinflammatory cytokines, e.g., IL-17 and IL-23. These proinflammatory cytokines are usually associated with chronic inflammation, critical for carcinogenesis, and tumor progression [107]. In addition, IL-10 is capable of promoting CD8+ T cell memory development, proliferation, and cytotoxicity. IL-10 has also been reported
to have potent antitumor effects by inhibiting macrophages, suppressing angiogenic factors, and activating CD8$^+$ T cell cytotoxicity [107]. A pegylated IL-10 has been clinically trialed and tumor regression occurred in four out of 15 renal cancer patients [108]. Patients that have activated intratumoral CD8$^+$ T cells, elevated IFN-γ and Granzyme B levels had diminished cancer growth [109].

2.7. TLR Activation Induces Immune Response against Cancer. A number of TLR ligands have been investigated as therapeutic agents in cancer immunotherapy or as cancer vaccine adjuvants. Three agents have been licensed by the FDA for clinical use [110]. These include bacillus Calmette-Guérin (BCG), monophosphoryl lipid A (MPL), and imiquimod which engages TLR2/TLR4, TLR4, and TLR7, respectively. Some of the TLR agonists that have been tested in clinical trials are shown in Figure 4.

2.7.1. TLR2. BCG is an attenuated strain of *Mycobacterium bovis* and is used to prevent the spread of human tuberculosis. BCG can engage TLR2 and TLR4 via several bacterial cell wall components to activate the MyD88 and NF-κB signal pathway. This leads to enhancement of TNF-α production which promotes innate immunity. It has been approved by the FDA for intravesical immunotherapy of bladder cancer [111]. BCG instillation for nonmuscle invasive bladder cancer after transurethral resection is regarded as the gold standard of therapy [112]. The activation of TLR2 on effector immune cells contributes to the immune response against cancer. A randomized controlled trial in advanced NSCLC patients (stage 3B or 4) using a TLR2 ligand CAD1-05, taxane and platinum chemotherapy compared to taxane and platinum chemotherapy showed no increased overall survival (OS). A subgroup analysis showed that squamous NSCLC patients had an improved median survival (hazard ratio, 0.55; 95%
Finally, the results suggest that it was not therapeutically effective [123].

A recent study in patients with late-stage indolent non-Hodgkin’s lymphomas explored the use of an in situ vaccine involving three phases: (1) a Fms-like tyrosine kinase 3 ligand to recruit intratumoral DCs; (2) local radiotherapy loading DCs with tumor antigens; and (3) TLR3 agonist poly (ICLC) activating DCs. Eight out of 11 cases had partial or complete regression of the treated tumor. Abscopal effect, i.e., distant untreated tumor regression upon local treatment was seen. Three out of 11 had significant (50% or greater) distant untreated tumor regression. Six out of 11 had distant untreated stable disease or minor (less than 50%) regression. Addition of PD-1 blockade to this vaccine increased the durable remission rate from 40% to 80% although PD-1 blockade alone did not produce durable remission in these patients. This vaccine induced cross-presenting DCs, upregulation of checkpoint molecules, and tumor-specific CD8⁺ cytotoxic responses [30].

The antineoplastic effects of anthracyclines are derived from activating endosomal TLR3 in tumor cells to produce type I IFN [119]. An in vitro study suggests that TLR3 signaling induces caspase-3 activation in lung adenocarcinoma thus promoting cancer cell apoptosis. Engagement of TLR3 is also capable of activating CD103⁺ lung DC subset that processes antigens derived from apoptotic cancer cells. Such a process would facilitate cross-priming of CD8⁺ T lymphocytes [120]. Downregulation of TLR3 expression enables liver cell carcinogenesis. It also prevents apoptosis in transforming precancerous hepatocytes [121].

2.7.3. TLR4. TLR4 has been investigated as an adjuvant in cancer vaccines and immunotherapy. The TLR4 agonist MPL is a derivative of LPS. It has similar immunostimulatory activity as LPS but with reduced toxicity. It is the only TLR ligand present in HBV and HPV vaccines [122]. MPL has the potential to elicit a potent anticancer immune response. Various cancer vaccine studies that involved more than 300,000 individuals have used a variety of adjuvants combined with MPL. Unfortunately, the results suggest that it was not therapeutically effective [123].

A recent study showed that paclitaxel-induced TLR4 signaling was essential for DAMPs production. This led to immunogenic cell death in ovarian cancer [124]. TLR4 activation in TP53 wild-type breast cancer cells induces IFN-γ production. The consequent downstream IFN-γ signaling and nuclear p21 activation inhibits tumor cell growth. In contrast, TLR4 activation in TP53 mutant breast cancer cells increases the secretion of several progrowth cytokines, e.g., IL-6, IL-8, and chemokine CXCL1. It decreases the production of costimulatory molecule CD154. Consequently, TP53 mutant breast cancer growth was promoted. Thus, TP53 may mediate the anticancer effects of TLR4 activation [125].

2.7.4. TLR5. Engagement of TLR5 in immune cells can act as a potent immune adjuvant. An engineered antibody was constructed by genetically fusing bacterial flagellin to a DC-targeting αCD40 antibody with incorporated leukemia-specific antigen. This antibody when loaded onto immature DCs and cocultured with T cells significantly increased IFN-γ and TNF-α-producing CD8⁺ T cells. Engagement of TLR5 improved the efficacy of this recombinant molecule [126]. A self-activating TLR5 signaling cassette composed of human TLR5 with a flagellin derivative has been employed in an adenovirus vector. The vector enters tumor cells and affects genes involved in inflammation. Concomitantly, mobilization of innate immune cells, e.g., neutrophils and NK cells involved in innate immunity into tumors occurred. This suppressed tumor growth and metastasis in a transgenic prostate cancer mice model. Mice vaccinated with irradiated prostate cancer cells transfected with this virus vector were protected on subsequent tumor challenges [127]. Nude mice implanted with TLR5- or MyD88-deficient human colon cancer cells displayed a dramatically enhanced tumor growth and inhibited tumor necrosis. Activation of TLR5 by flagellin injection peritumorally induced tumor necrosis and regression [128]. Vaccination of mice with liposomes bearing antigen and flagellin-related peptides induced potent suppression of tumor growth and metastasis. In the murine model, complete regression occurred in nine out of 10 mice challenged with syngeneic tumor cells [129]. Similar results were reported when flagellin was used as a tumor vaccine adjuvant in mice [130].

It has been observed that in patients with colorectal cancer, single-nucleotide polymorphisms in TLR5 modulate signaling responses to flagellin. The single-nucleotide polymorphism rs2072493/N592S in the TLR5 gene correlated with poor survival while rs5744174/F616L correlated with increased survival. In the TLR5 616LL homozygote carrier, there were increased mRNA levels of IL-6 and IL-1β mRNA in primary immune cells. This suggested that carcinogenesis of IL-6 might be relevant to flagellin induced TLR5-genotype dependent signaling [131].

2.7.5. TLR7. Imiquimod, a TLR7 agonist, is the only FDA-approved TLR agonist anticancer agent. It is used for the treatment of superficial basal cell carcinoma [132]. Topical application of imiquimod activates the TLR7 signaling pathway inducing production of proinflammatory cytokines, e.g., IFN-α, TNF-α, IL-6, and IL-8. These cytokines stimulate the innate and adaptive immune responses with significant anticancer effects [133]. In a clinical trial using 5% imiquimod
cream for the treatment of patients with breast cancer metastases affecting the skin, three out of 10 patients did not respond to treatment. Five patients had stable disease, i.e., the tumor did not progress or regress. Two patients had tumors that regressed. Intratumorally, lymphocyte infiltration and cytokines production occurred [134].

In mice with breast carcinoma subcutaneously implanted, 5% imiquimod cream inhibited tumor growth. This was accompanied by increased tumor infiltration by CD11c+, CD4+, and CD8+ cells. The combination of topical imiquimod and radiotherapy resulted in complete tumor regression in four out of six mice. In contrast, monotherapy with either imiquimod or radiotherapy failed to achieve complete tumor regression in any mice. In the four of the mice that had complete tumor regression, recurrence of the tumor occurred within 20–30 days after imiquimod discontinuation. Upon incorporation of cyclophosphamide into the regimen, three out of 14 mice remained tumor-free 65 days after cessation of imiquimod. On challenge with tumor cells, all three mice remained tumor-free 2 months after the challenge [135].

The progression of breast cancer is correlated with circulating MDSCs. Inhibition of STAT3 and activation of TLR7/8 had multiple effects. It can induce MDSCs repolarization and differentiation into mature myeloid cells. It can reduce the immunosuppressive activity of patient-derived MDSCs. It can decrease human breast cancer cell proliferation [136]. Such MDSCs repolarization also occurs in colorectal cancer. Resiquimod, a chemical analog of imiquimod, can activate TLR7/8 [137]. Activated TLR7/8 instructs MDSCs to differentiate into tumoricidal M1-macrophages. In the mice colorectal cancer model, it also reverses oxaliplatin resistance [34].

2.7.6. TLR8. Maintenance of immune homeostasis by regulatory T cells (Tregs) is achieved by inhibiting an excessive immune response. Decreased or enhanced Treg cell function can promote autoimmunity or carcinogenesis [138]. The combination of topical imiquimod and radiotherapy failed to achieve complete tumor regression in any mice. In the four of the mice that had complete tumor regression, recurrence of the tumor occurred within 20–30 days after imiquimod discontinuation. Upon incorporation of cyclophosphamide into the regimen, three out of 14 mice remained tumor-free 65 days after cessation of imiquimod. On challenge with tumor cells, all three mice remained tumor-free 2 months after the challenge [135].

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2.7.7. TLR9. Accumulating evidence has suggested that TLR9 activation is a promising therapy for a variety of cancers [74]. TLR9 can induce inflammation by binding to DNA fragments released from cancer cells killed by chemotherapy. In breast cancer patients that received neoadjuvant chemotherapy, increased OS was correlated with elevated TLR9 expression in cancer cells [145].

Lefotilimod, a DNA-based TLR9 agonist, was used in a phase II clinical trial on small cell lung cancer. It was trialed as maintenance treatment in patients with extensive disease. In patients that positively responded to chemotherapy, post-treatment maintenance of lefofilimod administration was used with the aim of trying to extend the duration of disease control. Analysis using the criteria of the intent-to-treat population found that lefofilimod did not improve OS and PFS. However, in patients with decreased number of activated CD8+ CD19+ B cells or those with a history of chronic obstructive pulmonary disease, improvement of OS was observed [146]. Thus, lefofilimod administration, as adjunctive follow-on immunotherapy after chemotherapy, deserves further investigation in certain subgroups of patients. Lefotilimod maintenance was also conducted in patients with metastatic colorectal carcinoma. It was well tolerated and significantly improved the PFS of patients that had disease control after standard first-line chemotherapy [147]. Potent type I IFN response in colonic mucosa is present and may contribute to better survival [148].

Pembrolizumab is a PD-1 monoclonal antibody. SD-101 is a synthetic CpG oligodeoxynucleotide (ODN) capable of activating TLR9. These two agents were used in a clinical trial of patients with unresectable or metastatic melanoma. This trial included nine patients that did not have previous PD-1 treatment. The overall response rate, the estimated 12-month PFS and OS were 78%, 88%, and 89%, respectively. No severe
adverse side effects were observed. In 13 patients who had prior anti-PD-1 therapy, the overall response rate was 15%. Increased numbers of CD8+ T cells, NK cells, cytotoxic lymphocytes (CTLs), DCs, and B cells were detected in cancer tissue [149]. Intratumoral administration of SD-101 combined with low-dose radiation in patients with untreated indolent lymphoma also showed promising antitumor effects. All 29 patients had tumor regression at the treated sites and 24 out of 29 cases had tumor regression at untreated sites. Among these 24 cases that showed the abscopal effect, five patients achieved a partial response and one patient had a complete response [150].

Another TLR9 agonist, EMD 1201081, also known as IMO-2055, did not display any clinical benefit in combination of cetuximab in recurrent or metastatic HNSCC patients [151]. In a phase III NSCLC trial, stage IIIB or IV patients were randomized to two treatment groups. One group received gemcitabine and cisplatin. The other group received gemcitabine, cisplatin, and TLR9 agonist PF-3512676. In the first interim analysis, the median OS and median PFS were not significantly different between the two groups. Adverse hematologic events classified as being grade 3 or greater were more numerous in the PF-3512676 arm. The study was halted due to the occurrence of adverse events and low therapeutic efficacy [152]. Another phase III NSCLC trial using standard paclitaxel/carboplatin chemotherapy alone or in combination with PF-3512676 also did not show improved OS or PFS in the PF-3512676 arm [153].

2.8. Regulation of Elements in the TLR Signaling Pathway. Aberrant activation of the NF-κB-induced colonic neoplasm, application of an IRAK4 inhibitor, PF06650833, significantly inhibited carcinogenesis. Treatment of cancer cells with oxaliplatin or 5-FU resulted in upregulated TLR9 expression and enhanced IRAK4 and NF-κB activities. This provided a feedforward mechanism to protect cancer cells. In colorectal cancer patients, the enhanced tumor expressions of phosphorylated IRAK4 and its mRNA are correlated with worse outcomes [154]. IRAK1 and IRAK4 were upregulated in HCC patients. IRAK1 can induce oncogenesis. In vitro, treatment of HCC cells with sorafenib resulted in increased IRAK1 and IRAK4 expressions. The sensitivity of HCC cells to sorafenib was increased by inhibition of IRAK1 [155]. Serine/threonine-protein kinase 4 is a tumor suppressor. It associates with IRAK1 and leads to IRAK1 phosphorylation and degradation. This culminates in the inhibition of TLR4/9-induced proinflammatory cytokine secretion and promotion of TLR3/4-induced IFN-β production. In a murine model, when this kinase was knocked out in macrophages, enhanced oncogenesis of chronic inflammation-associated HCC occurred [156]. Thus, regulation of TLR signaling in cancer by targeting IRAK4/IRAK1 may improve the efficacy of chemotherapy and prognosis.

V-domain immunoglobulin suppressor of T-cell activation (VISTA) is a novel negative checkpoint ligand. Similar to PD-L1, VISTA suppresses T-cell activation. VISTA is expressed on myeloid cells and Treg cells but not on tumor cells. VISTA suppresses the phosphorylation of inhibitor of NF-κB (IκB) kinase (IKK) α/β and the degradation of IκB. Deletion of VISTA in macrophages enhanced polyubiquitination of TNF receptor-associated factor 6 (TRAF6) and promoted downstream signaling. This results in increased TLR-mediated proinflammatory cytokine production. Blockade of VISTA can enhance the proinflammatory immune response triggered by TLR agonists, reprogram tolerogenic myeloid cells, and promote T-cell-mediated anticancer immunity [157].

2.9. DAMP–TLRs Interactions Affect Carcinogenesis. DAMPs are endogenous molecules released by dying cells or by the extracellular matrix of the host when damage occurs. Similar to PAMPs that originate from microorganisms, host endogenous DAMPs are also capable of inducing early innate and adaptive immune responses. These are referred to as the signal 0 event [158]. DAMPs can bind to a number of PPRs including TLRs to induce inflammation and, thus, involve in carcinogenesis.

DAMPs release is either passive or active. Passive release is when the integrity of the cell membrane is ruptured and DAMP molecules are leaked. This implies cellular death. Passive release is agnostic to the mechanism of cell death, e.g., necrosis, necroptosis, apoptosis, pyroptosis, ferroptosis, and extracellular traps [159].

The process of DAMPs originating from living cells is termed active release. Many DAMPs are unable to be secreted via the traditional secretory pathway composed of ER and Golgi apparatus. The inability to use the traditional pathway is because DAMP molecules are nucleotides or proteins lacking a signal peptide. The active release process is largely represented by exocytosis consisting of secretory lysosomes and exosomes. The released DAMP molecules can be recognized by a variety of receptors, e.g., TLRs, NLRs, receptor for advanced glycation end products (RAGE), triggering receptors expressed on myeloid cells (TREM) and P2X receptors (P2XRs). Upon binding these receptors, DAMPs induce cytokine and chemokine productions and inflammatory responses. Such responses in turn induce further release of DAMPs [159].

DAMP–TLR interaction was discovered more than 2 decades ago. A member of the DAMP family, heat shock protein 60 (HSP60) was found bound to TLR4 in macrophages. This induced the production of TNF-α and NO [160]. Subsequently, a number of DAMPs have been discovered to engage in different TLRs to induce host immune response and inflammation. For example, cell surface TLR2 dimerizes with TLR1 or TLR6 to form a heterodimer and recognizes a number of DMAP molecules, e.g., HMGB1, β-defensin-3, monosodium urate, pancreatic adenocarcinoma upregulated factor, serum amyloid A, neutrophil elastase, HSP60, HSP70, glycoprotein 96 (gp96), surfactants A/D, eosinophil-derived neurotoxin, biglycan, hyaluronic acid, MMP2, and versican [161]. TLR4 recognizes HMGB1, high-mobility group nucleosome-binding protein 1 (HMGN1), gp96, HSP22, HSP60, HSP70, HSP72, HSP90, hyaluronan, heparan sulfate, fibrinogen, monosodium urate, pereoxiredoxin, biglycan, neutrophil elastase, serum amyloid A, oxidized LDL, fibronectin EDA, fibrinogen, tenascin-C,
lactoferrin, β-defensin-2, saturated fatty acids, and surfactant protein-A [161]. Endosomal TLR3 recognizes tumor-derived dsRNA and siRNAs. TLR7 and TLR8 recognize tumor-derived ssRNA and siRNAs, antiphospholipid antibodies, and miRNAs. TLR9 recognizes tumor mtDNA, HMGB1, and IgG.

Cell transformation is the initial step in oncogenesis. The innate immune cells of the host recognize these transformed cells and destroy them. This results in the release of DAMP molecules. The released DAMP molecules interact with TLRs in infiltrating immune cells. DAMPs also interact with cancer cells to modulate cell apoptosis, necrosis, migration, and metastasis [162]. This also initiates a positive feedback cycle of DAMP molecules release, ultimately resulting in increasing cytokine levels with, e.g., IL-6, IL-10, IL-12, TGF-β, and TNF-α. These molecules cause tumor elimination or escape [161].

The released DAMPs can also bind to TLRs and other PRRs to initiate an inflammatory reaction. Such inflammation results in a few cells losing cell cycle control and cellular contact inhibition. These transformed cells then attach to the extracellular matrix and proliferate uncontrolledly resulting in a clinical presentation of tumor [41, 161]. With such growth, hypoxia and nutrient deficiency in the central area of the tumor eventually results. This leads to cell degeneration and necrosis. In addition, immunogenic cell death occurs concurrently. Similar to TLRs activation, paradoxical effects of DAMP molecules are present. The interaction of DAMPs and TLRs induces a series of chemokines that recruit immune cells to the tumor. These immune cells are either activated to exert anticancer effects or transformed into suppressive phenotypes to promote tumor growth. For example, a variety of HSP molecules released by tumor cells under heat stress can induce tumor cells to produce chemokines including CC chemokines, CXC chemokines, CX3C chemokines that chemo-attract DCs and T cells. This causes an increase of the infiltration and activation of DC and T cells, decrease of infiltration of Treg and MDSC cells in the tumor. Interaction of HSP70 and TLR4 is essential for the activation of DCs to elicit an immune response against cancer [163]. On the other hand in a mouse prostate cancer model, HMGB1-activated tumor-specific T cells were found to infiltrate into the tumor. Such infiltrating T cells express lymphotoxin α1/2 and recruit macrophages. Both events promote tumor growth. Unsurprisingly, antibodies that block HMGB1 reduced the number of infiltrating T cells and macrophages impeding carcinogenesis [164].

DAMP molecules such as HMGB1 and HSP90 when released extracellularly recruit TAMs and tumor-associated DCs and induce an increased expression of T cell immunoglobulin and mucin domain protein-4 (TIM-4). The increased amount of TIM-4 interacts with adenosine monophosphate activating kinase-α1 to activate autophagy-mediated digestion of engulfed tumor cells. An effect of such tumor cell death is reduced TAA presentation and diminished CTL response. Blockade of TIM-4 and adenosine monophosphate activating kinase-α1 mediated autophagy can reverse this impaired immune response [165].

HMGB1 is the most widely researched DAMP molecule. It is also the most abundant DAMP molecule released by dying tumor cells. Thus, HMGB1 is highlighted below as an example to illustrate the involvement of DAMP molecules in cancer.

HMGB1 is overexpressed in tumor endothelium and intimately involved in carcinogenesis [166]. It influences cell migration, adjusts cellular adhesion, modulates the extracellular matrix components [167], and activates vascular endothelial cells to promote neovascularization [168].

HMGB1 in human HCC-derived exosomes stimulates TIM-1+ regulatory B cells expansion and production of IL-10 to inhibit anticancer CTL response via TLR2/4-mitogen-activated protein kinase pathway [169]. Irradiated breast cancer cells release HMGB1 to interact with TLR4 in macrophages. HMGB1 also promotes macrophage differentiation toward the M1 phenotype. The differentiated macrophages secrete TNF-α capable of inhibiting proliferation and migration of nonirradiated cancer cells. This facilitates radiation-induced abscopal effect [170]. Another study showed that HMGB1 can be released from glioblastoma treated with combined immunotherapy with cytotoxic agents. HMGB1 induced TLR2-dependent tumor regression through activation of DCs and promotion of cross-presentation. Blockade of HMGB1 abolished the therapeutic efficacy [171].

HMGB1 can induce inflammatory responses through a number of mechanisms including maturation of DCs, increased expressions of costimulatory molecules, MHC-II and production of proinflammatory cytokines, such as IL-12 and IFN-γ. All these stimulate naïve CD4+ T cell to differentiate into Th1 phenotype [172]. HMGB1 can stimulate neutrophils and monocytes to secrete cytokines. These cells can also migrate to an inflamed region [173]. HMGB1 induces endothelium cell to express TNF-α and monocyte chemotactic protein-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 to adhere to inflammatory cells present in inflamed tissue [174]. Within the cancer cell, HMGB1 can activate TLR2 to promote cancer stem cell renewal, induce Treg cell proliferation, and facilitate resistance to chemotherapy [175]. HMGB1 also interacts with phosphatidyserine to inhibit phosphatidyserine-mediated phagocytosis of apoptotic cells. This results in necrosis releasing cellular contents causing an exacerbation of inflammation [167]. In a mouse colorectal cancer model, gastermin E-mediated pyroptosis induced HMGB1 release and promoted carcinogenesis via ERK1/2 signaling [176].

HMGB1 is capable of interacting with other molecules, such as nucleic acid, IL-1β, and LPS to modify its intrinsic functions. For example, HMGB1 combined with CpG can induce increased IFN-α production in mouse bone marrow-derived plasmacytoid DCs [177]. HMGB1 is also capable of binding TLR9, without combining with CpG-DNA, to induce inflammatory cytokine production [177, 178]. Stimulation of HMGB1-deficient DCs with CpG-DNA induces
less production of IFN-α/β [178]. HMGB1-nucleosome complex released from apoptotic cell can activate DCs and macrophages and produce inflammatory cytokines through TLR2-MyD88 signaling [179]. HMGB1 is also able to associate with TLR4-expressing cells to induce enhanced cytokine production through TLR4 [180].

HMGB1 binds to TLR4 in cancer cells to induce TGF-β production. TGF-β then promotes the expression of immunosuppressive protein galectin-9 which enables immune evasion of cancer cells in an autocrine manner. In cancer cells without TLR4, HMGB1 can engage with TLR4-expressing myeloid cells in TME to induce the immune evasion in a paracrine pattern [181]. This suggests that HMGB1 is involved in tumor growth and progression. However, in vitro and in vivo experiments with CT26 colon cancer cells (syngenic from BALB/c mice), TS/A breast cancer cells (syngenic from C57BL/6 mice), EG7 cells (OVA-transfected EL4 cells), MCA205 fibrosarcoma cells, and the Glasgow osteosarcoma suggested that interaction of HMGB1 and TLR4 plays a role in DCs activation and cross-presentation to promote CTL response against cancer cells [182].

2.10. Artificial Intelligence Promotes Research on TLRs and Cancer. Artificial intelligence (AI) is already being used in some cancer diagnosis. It also serves as an aid in treatment and prognoses [183, 184].

There are a variety of techniques present in AI. Currently, the most commonly used AI technique in medical research is machine learning. Machine learning can be divided into three categories, algorithms that use supervised learning, algorithms that use unsupervised learning, and reinforcement learning (Figure 5) [185]. In unsupervised learning, the data are not classified [184]. In supervised learning, the data are classified based on the characteristics present.

An example of the use of supervised learning is seen in a study where 28 different types of cancer were histopathologically assessed. Widespread associations between bulk gene expression levels and histopathology were found [186]. Another study employed an AI algorithm to classify miRNA sequences and secondary structural features. The predicted miRNAs that involved in TLR7/8 activation were validated by laboratory experiments. There was a high degree of agreement between AI predictions and the results of experiments conducted in the laboratory. This validated the algorithm used [187]. Thus, this algorithm assisted in computer-based structure-based screening of various oligonucleotides for TLRs ligands or inhibitors.

IRAK1 is a downstream element of TLR signaling pathway. Suppression of IRAK1 exhibited activity against myelodysplastic syndrome and certain subtypes of acute myeloid leukemia. Traditional methods based on discriminatory structure-based virtual screening have been used to discover IRAK1 inhibitors. Using this approach, in a study looking at IRAK1 inhibitors, only one compound was discovered. In contrast, using an AI system which combined molecular docking, pharmacophore scoring, and molecular descriptors, four compounds were identified. Additionally, these exhibited good activity against IRAK1 [188].

Another example of the application of AI techniques is a study where 6,000 ODNs were randomly generated and screened by combining repeated random downsampling and random forest algorithms. This approach was used as screening of the TLR9 ligand, a single-stranded DNA ODN contains unmethylated CpG motifs that have numerous rotatable bonds. If a traditional structure-based virtual screening of CpG ODNs was used, this would be time consuming and more expensive. Of the 6,000 ODNs analyzed, 100 ODNs were selected based on the combination of repeated random downsampling and random forest algorithms screening. These were then synthesized and tested in the laboratory. Ninety-one of the 100 ODNs displayed high activity with TLR9 [189]. The algorithm used is shown in Figure 6.

The efficacy of machine learning is also seen with an alkaloid isolated from a traditional Chinese medicine, Gentianine. A machine learning algorithm predicted that the anti-inflammatory effects were mediated by inhibition of TLR4 signaling. This algorithm was based on network pharmacology and molecular docking. One thousand forty-nine pharmacologically active protein schemas were initially downloaded from Drugbank. Then using a sequential selection algorithm and a molecular docking programing technique, these proteins were mapped to ischemic stroke targets in Genbank. Seventeen proteins that overlapped were obtained. These proteins were then tested for protein–protein interaction using GENEMANIA. Assessing all the target proteins, the highest total protein–protein interaction weight was 59.58% obtained with TLR4. This suggested an important role for TLR4. Additionally, enrichment of TLR and NF-κB signaling pathways was shown by using the Kyoto Encyclopedia of Genes and Genomes pathway analysis. Gene ontology enrichment analysis also demonstrated enriched protein binding and NF-κB regulation. These in silico results suggested that the mechanism of Gentianine was suppression of the NF-κB pathway mediated by TLR4. In conclusion, this particular machine learning schema was verified by traditional approaches [190].
Another example of machine learning algorithm used in combination with additional mathematical/statistical techniques is highlighted with a study of rheumatoid arthritis patients to predict the gene polymorphisms of TLR4 and TLR9. The algorithm incorporated multivariate logistic regression, elastic net regression, random forest, and support vector machine. It was found that patients with TLR9 polymorphism (rs352139) had better therapeutic responses to TNF-α inhibitors [191].

A branch of machine learning called deep learning has been a focus of attention. Deep learning is a multilayered network structure that mimics the neurological architecture of the brain (Figure 7), a neural network. Neural networks can handle hundreds of millions data points/examples to train a particular algorithm within an existing network of connections numbering in the billions (Figure 8) [192].

Deep learning has been explored by endoscopists to detect abnormal lesions. It has also been used to predict malignant lesions in the gastrointestinal tract [193]. Another example highlighting the application of this technique is humanized immunoglobulin G4 monoclonal antibody against TLR2, Tomaralimab. This was developed to inhibit abnormal activation of TLR2 in diseases such as inflammation associated with ischemia/reperfusion-induced tissue damage. With the construction of a Tomaralimab homology model and its complex with TLR2 with different epitopes, a novel epitope was predicted. This could be used as a basis to construct therapeutic antibodies. A geometric deep learning algorithm revealed that epitope-mutated alanine substitutions significantly affected the affinity of Tomaralimab with mutated epitopes. Thus, this particular algorithm was able to identify and predict changes in the complementarity-determining region and promote the antibody’s efficacy [194].

Drug-resistance has been predicted [195] and new drugs designed using AI [196]. Indeed, novel drugs/proteins with defined functions that do not exist in nature can be visualized. Using reinforcement learning, variational inference, and tensor decompositions by a generative two-step machine learning algorithm, potent inhibitors of discoidin domain receptor 1 (DDR1) have been discovered. In a study, six datasets were initially processed to exclude outliers and reduce the number of compounds that contained similar structures. Then, this particular AI algorithm was trained on three datasets derived from the initial six datasets. The initial output consists of approximately 30,000 structures. This was then filtered and structurally evaluated by the general and specific kinase self-organizing maps and pharmacophore modeling. These evaluation modalities were based on crystal structures of compounds in complex with DDR1. The values of molecular descriptors and root-mean-square deviation (RMSD) were calculated. Using RMSD values at a certain cutoff, 40 structures were selected. Thirty-nine of the 40 structures obtained were not in any published patent(s) or application(s).

Six of these 40 compounds were then synthesized for laboratory validation. Four compounds displayed strong to moderate potency for DDR1 inhibition. Two compounds with strong inhibitory potency were validated in cell-based in vitro experiments. The leading compound was tested in mice with favorable pharmacokinetic results. Quantum mechanical analysis demonstrated that the leading compound formed multiple hydrogen bonds and had favorable charge and hydrophobic interactions with the active site residues of DDR1 kinase. The design, synthesis, and experimental validation of novel molecules targeting DDR1 kinase took 46 days. This period of time for the discovery process has been significantly reduced [197].

To accelerate research, a TLR database (TollDB) has been constructed (https://tolldb.drug-design.de) and is freely accessible to the public. It contains all the reported small organic molecules targeting TLRs and their assay conditions. As of December 2023, there are "4,925 datapoints describing 2,155 compounds tested in 36 assay types with 553 distinct assay conditions" (https://tolldb.drug-design.de). Databases with imbedded machine learning techniques are also available for researchers [198].

A clinical trial is now taking place using a drug designed completely by AI, INS018_055, for the treatment of idiopathic pulmonary fibrosis [199]. The principles behind the
design of this drug are detailed in nature [200]. It is not inconceivable that AI techniques could be used to design a novel TLR drug for clinical trials.

Very recentlyChat-GPT4, a large language model, has exploded on the scene. This has significant implications not only for medical practice but also for research [201]. Chat-GPT4 is based on deep learning principles. An open edition of another large language model created by Facebook is freely available. The development of such technology has been very rapid. The latest and current version (as of June 2023), ORCA based on the model created by Facebook is even more powerful than Chat-GPT4 that appeared in early 2023 [202].

### 3. Discussion and Conclusion

#### 3.1. Heterogeneity of Cancer and TLR Expression Influences the Results of TLR Activation.

The TLR family is the most important PRRs that regulate innate immunity against cancer cells. However, modulation of TLRs is not fully understood. TLRs are involved in cancer promotion and rejection (see Appendix Table S2). Such conflicting outcomes appear to be dependent on the type of cancer [203]. Even within the same type of cancer, in addition to cellular nonhomogeneity, heterogeneity is present with different proportions of cellular components, e.g., tumor infiltrating lymphocytes, macrophages, and fibroblasts. These may possess different TLR expression patterns and respond differently to TLR agonists [5].

It is intriguing that cancer cells from the same histological origin have distinct TLR expression and response patterns. For example, prostate cancer cell lines LNCaP, PC3, and DU145 express TLRs differently. LNCaP does not express TLR7 and TLR8 genes, DU145 does not express TLR4 gene, and PC3 does not express TLR7 gene. All three cell lines express TLR2 protein. TLR4 protein can be detected in LNCaP and PC3 but not in DU145. All detectable TLR2 and TLR4 are localized perinuclearly. They also react to LPS differently [204]. These results suggest that cancer cells, even in a
histologically identical cancer, may have different TLRs expressions. Their responses to TLR modulators may be different.

3.2. TLR Expression Patterns and Other PRRs Are Potentially Confounding Factors Influencing Study Results. Some TLR ligands may be able to activate PRRs that are not TLRs. For example, TLR3 is not the sole receptor of poly (I:C). Other receptors, e.g., melanoma differentiation-associated gene 5 (MDA5) or NLRP3 can be activated by poly (I:C). Such activations can result in either proinflammatory cytokines production, type I IFN production, or inflammasome formation [205]. Therefore, the oncogenic effects of TLR ligands induced in cancer cells may not be solely attributed to TLR activation. Endosomal TLR9 has been found predominantly expressed on the cell surface in some cancer cell lines. Such an expression pattern of TLRs in cancer cells may imply that their signal pathways or the biological effects are different from those in immune cells. Stimulation of these aberrant expressed TLRs in cancer cells may promote tumor growth.
and invasion [206]. Additionally, TLR polymorphisms in cancer cells may be different from those present in immune cells. Polymorphisms and haplotypes of TLR4 and TLR9 affect patients’ susceptibility to cervical cancer [207]. TLR2 polymorphisms are associated with an increased risk of oncogenesis [208]. The constitution and haplotype of TLR in cancer cells may be different from those in immune cells. Due to this difference, the effects of TLR activation in cancer cells and immune cells may be different.

3.3. Intratumoral Delivery of TLR Agonist Elicits More Potent Anticancer Immunity. TLR agonists in combination with chemotherapy and/or radiotherapy may enhance anticancer effects. Conventional radiotherapy or chemotherapy destroys cancer cells and also enhances the expression of TAAs. These modalities of therapy and the release of TAAs target tumor stroma, Treg cells and activate effectors of innate immunity through a TLR-dependent mechanism [209]. The killed cancer cells release DAMPs to activate innate immune cells via TLRs signaling, e.g., DCs. These activated innate immune cells take up the TAAs released by killed cancer cells to cross-prime immunoreactive lymphocytes eliciting cancerspecific cell immunity: immunorejection. As TAAs are more concentrated in the tumor, intratumoral delivery of TLR agonist may increase anticancer efficacy. The delivery route can cause an abscopal effect [30].

3.4. TLR Antagonists in Cancer Immunotherapy Are Another Avenue for Further Investigation. Some reports have suggested that activation of TLRs may induce oncogenesis. It seems reasonable to use TLR antagonists to block TLR signaling and thus retard cancer development. However, most of the TLR antagonists in clinical trials are in inflammatory diseases or in autoimmune disorders [210]. A few of these TLR antagonist agents have been investigated for their effects against cancer in vitro or in animal models [211]. For instance, TAK-242 (resatorvid) which inhibits TLR4 does this by binding to the intracellular domain of TLR4. This inhibits the association of TLR4 with its adaptor TIRAP or TRAM to suppress downstream signaling [212]. In the murine colitis-associated colon cancer model, application of TAK-242 during the inflammatory phases strongly diminished the development of colonic cancer (Figure 9) [213]. TAK-242 also inhibited the proliferation of anoikis-resistant cells and suppressed the clonal growth in breast cancer and ovarian cancer cell lines [214, 215]. Inhibition of TLR7 and TLR9 signaling by their specific antagonist IRS-954 or by a nonspecific inhibitor, chloroquine, in the hepatocellular carcinoma cell line suppressed cancer cell proliferation. Such intervention also inhibited tumor growth in the mouse xenograft model. Chloroquine treatment also suppressed the development of hepatocellular carcinoma in the diethylnitrosamine/nitrosomorpholine-induced rat model [216]. However, clinical trials have not validated the use of such compounds in humans.

3.5. Closing Comments. TLR activation in cancer affects TLR type-dependent, cancer type-dependent, and tumor individual-dependent processes. TLR activation is a double-edged sword in cancer either inducing immunotherapeutic effects or promoting EMT transformation, cancer cell proliferation, metastasis, and resistance to therapy (Figure 10). When using TLR ligands as anticancer agents, a balance between the expression of TLR type
in immune cells and the type of cancer cells requires careful consideration. A personalized treatment regimen should be based on the TLR expressive and responsive patterns for the specific tumor. Clinical trials using various combinations of therapeutic modalities would be desirable. AI is likely to provide additional insights into the role of TLRs in cancer [187].

Abbreviations

AI: Artificial intelligence  
AIM2: Absent in melanoma 2  
APC: Antigen-presenting cell  
BCG: Bacillus Calmette-Guérin  
CCL: Chemokine (C–C motif) ligand  
CpG: Unmethylated deoxyguanosine preceding deoxyguanosine  
CTL: Cytotoxic lymphocyte  
CXCL: Chemokine (C–X–C motif) ligand  
DAMP: Damage-associated molecular pattern  
DC: Dendritic cell  
DD: Death domain  
DDR1: Discoidin domain receptor 1  
dsRNA: Double-stranded RNA  
EMT: Epithelial-to-mesenchymal transition  
ER: Endoplasmic reticulum  
FADD: Fas-associated cell death domain

FIGURE 10: Activation of TLR is a double-edged sword in cancer. Cancer cells can release TAAs to trigger innate immunity including DCs maturation, TLR activation, and induce trained immunity. Activation of innate immunity induces inflammation. Persistent inflammation promotes cellular transformation and carcinogenesis. Activation of TLR in DCs induce DC maturation and present the relevant TAA epitope to CD4+ T cells in the presence of MHC-II and costimulation factors CD40 and CD40L. Naïve CD4+ T cells thus are activated as Th1 cells. Th1 cells then assist in the activation of CD8+ T cells and its transformation into CTLs to exempt cancer cell killing. DCs are capable of presenting the TAA epitope to CD8+ T cells with MHC-I in the presence of costimulation factors CD80/CD86 and CD28, i.e., cross-presentation. Cross-primed CD8+ T cells transform into CTLs and kill target cancer cells. However, activation of TLR in cancer cells induces increased expression of PD-L1, MMPs to facilitate cancer cell chemoresistance and metastasis. Cellular TLR activation from cancer cells also produces a variety of known and/or unknown factors to induce cancer stem cell proliferation, EMT transformation, and resistance to apoptosis. A number of immunosuppressor cells are also recruited and induced in the tumor microenvironment to prevent cancer cells from being eliminated by any immunological mechanism. Abbreviations: CTL, cytotoxic lymphocyte; DC, dendritic cell; EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloproteinase; PD-L1, ligand of programmed death 1; TAA, tumor-associated antigen; and TLR, toll-like receptor.
IFN: Interferon  
HNSCC: Head and neck squamous cell carcinoma  
HCC: Hepatocellular carcinoma  
gp130: Glycoprotein 130  
FDA: Food and Drug Administration  
IκB: Inhibitor of NF-κB  
IKK: IκB kinase  
IL-1R1: Interleukin-1 receptor 1  
IL-1RαcP: Interleukin-1 receptor accessory protein  
IRAK: IL-1R associated kinase  
IRF: Interferon regulatory factor  
IRAK-M: Interleukin-1 receptor associated kinase-M  
LPS: Lipopolysaccharide  
Mal: Myeloid differentiation factor 88-adapter-like  
MDSC: Myeloid-derived suppressor cell  
MHC: Major histocompatibility complex  
MDM2: Myeloid differentiation factor 2  
MD2: Myeloid differentiation factor 2  
Mal: Myeloid differentiation factor 88-adapter-like  
mtDNA: Mitochondrial DNA  
MMP: Matrix metalloproteinase  
MPL: Monophosphoryl lipid A  
MHC: Major histocompatibility complex  
MMP: Matrix metalloproteinase  
MHC: Major histocompatibility complex  
NAP: NF-κB activating kinase-associated protein  
NAP-β: NF-κB activating kinase-β  
NEMO: NF-κB-essential modulator  
NF: Nuclear factor-κB  
NK: Natural killer  
NLR: Nucleotide oligomerization domain-like receptor  
NLRP3: NLR pyrin domain 3  
NOD: Nucleotide oligomerization domain  
NSCLC: Non-small cell lung cancer  
ODN: Oligodeoxynucleotide  
OS: Overall survival  
PAMP: Pathogen-associated molecular pattern  
PD-1: Programmed death 1  
PD-L1: Ligand of programmed death 1  
PFS: Progression-free survival  
poly(I): Polynosinic–polycytidylic acid  
PRR: Pattern recognition receptor  
RIP: Receptor-interacting protein  
RMSD: Root-mean-square deviation  
SOCS3: Suppressor of cytokine signaling 3  
ssRNA: Single-stranded RNA  
STAT3: Signal transducer and activator of transcription 3  
TAA: Tumor-associated antigen  
TAB: Transforming growth factor β-activated kinase-1  
TAK: Transforming growth factor β-activated kinase-1  
TAK1: Transforming growth factor β-activated kinase-1  
TAM: Tumor-associated macrophage  
TBK: TRAF family member-associated NF-κB activating binding kinase  
Th: T helper cell  
TIM-4: T cell immunoglobulin and mucin domain protein-4  
TIR: Toll/IL-1 receptor  
TIRAP: TIR domain-containing adapter protein  
TLR: Toll-like receptor  
TME: Tumor microenvironment  
TNF: Tumor necrosis factor  
TRAF: TNF receptor-associated factor  
TRAM: TRIF-related adaptor molecule  
Treg: Regulatory T cell  
TRIF: TIR domain-containing adaptor inducing interferon-β  
VISTA: V-domain immunoglobulin suppressor of T-cell activation  

Conflicts of Interest  
The authors declare that there are no conflicts of interest regarding the publication of this article.  

Authors’ Contributions  
Yingxiang Yang and Chengyue Jin share first authorship.  

Supplementary Materials  
Appendix Table S1: registered clinical trials with TLR agonist/antagonist in cancer treatment (from https://clinicaltrials.gov/ as of July 2023). Appendix Table S2: the pro- and anti-cancer effects of TLR agonists. (Supplementary Materials)  

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