

# Emerging role of Toll-like receptor 4 in hepatocellular carcinoma

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**Abstract:** Toll-like receptor (TLR) signaling has been implicated in inflammatory-related cancers. The upregulation of TLR signaling in hepatocellular carcinoma (HCC) suggests that it may play an essential role in the prognosis of chronic and inflammatory diseases that ultimately culminate in HCC. Here, we provide evidence about the involvement of the TLR pathway in the initiation, progression, and metastasis of HCC. The differential expression of TLR in epithelial cells has also been discussed. In particular, we emphasize the physiological role of TLR4 in the development and pathogenesis of HCC and propose novel and promising approaches for HCC therapeutics with the aid of TLR ligands.

**Keywords:** hepatocarcinogenesis, inflammation, signal pathway, immune therapy

## Introduction

According to the World Health Organization, hepatocellular carcinoma (HCC) is a worldwide cause of cancer-related death second to lung cancer.<sup>1</sup> It is particularly prevalent in Southeast Asia and sub-Saharan Africa due to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and toxins in the food and water supply. Chronic hepatitis and cirrhosis caused by HBV and HCV infection are the major risk factors of primary HCC.<sup>1</sup> HCC can currently be treated only with surgery at the early and middle stages. The efficacies of chemotherapy and radiotherapy are far from satisfactory. Recurrence is quite common in patients with HCC who have had a radical resection, and the survival rate is 30%–40% at 5 years postsurgery.<sup>2</sup> This is because those malignant cells developed intricate mechanisms that enable them to inhibit immune cells through the secretion of specific cytokines that create an immunosuppressive environment.<sup>3</sup> Although many factors are involved in the pathogenesis and progression of HCC, its underlying mechanisms remain unknown. Recent research demonstrated that Toll-like receptor (TLR)4 expression levels were strongly associated with microvascular invasion and early recurrence.<sup>4</sup> It has been reported in murine models that specific HBV or HCV proteins can induce HCC without other oncogenic alterations.<sup>5</sup> The excessive compensatory proliferation of differentiated hepatocytes caused by chronic liver injury is thought to be one of the major pathogenic mechanisms underlying HCC development.<sup>6,7</sup>

Innate immunity that is conserved through evolution represents the first line of protection against microbial pathogens. Innate immunity discriminates and eventually clears a variety of pathogens and their nonpathogenic symbiotic bacteria via pattern-recognition receptors such as TLRs. TLRs detect invading microbes and recognize

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intracellular anomalies to which they mount an immune response, thereby playing a cardinal role in the homeostasis of the human immune system.<sup>8,9</sup> As an important pathogen recognition molecule, TLRs activated in the immune cells play a critical role in the host's defense and eventually lead to adaptive immune responses.<sup>10</sup> On the other hand, TLR stimulation can also directly or indirectly enhance the immunosuppressive function of regulatory T-cells that favors tumor development.<sup>11-13</sup> It was previously thought that TLR was mainly expressed in immune cells. However, emerging evidence suggests that TLRs are expressed in some types of tumor cells, and that signaling of TLRs can promote tumor proliferation and survival, immune evasion, and drug sensitivity.<sup>14,15</sup> The endogenous ligands released by cellular stress or apoptotic cells can activate TLR4 and lead to aseptic inflammation. Abnormal TLR activation can jeopardize normal physiological processes and cause several inflammatory diseases, cancers, and autoimmune diseases.<sup>16,17</sup> Research over the past decade has focused on elucidating various TLR-associated functions, intermediate molecules, and ligands. There is a well-established link between TLR-induced inflammation and cancer development and progression.<sup>18,19</sup> Similarly, TLRs are known to play a vital role in HCC. In this review, we will focus on reports concerning TLR4 signaling and its involvement in HCC development and progression, as well as on the therapeutic benefits that could arise from TLR4 stimulation.

## TLR receptors, ligands, and signaling pathways

TLRs are a family of transmembrane receptors that play a key role in innate immunity. TLRs prevent pathogenic invasion by recognizing pathogen-associated molecular patterns (PAMPs), which are highly conserved components derived from a range of microbial agents, as well as endogenous macromolecules released by injured tissue.<sup>20</sup> To date, ten TLRs have been identified in humans. TLR localization is heterogeneous and varies from the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, and mouse TLR11 and TLR12) to the endosomes (TLR3, TLR7, TLR8, and TLR9),<sup>21</sup> according to PAMP localization. The two most eagerly studied TLRs are TLR2 and TLR4, the pattern-recognition receptors for gram-negative and -positive bacterial products, respectively. TLR4 responds to various invading exogenous pathogens through PAMPs and recognizes the endogenous ligands from necrotic cells through damage-associated molecular patterns (DAMPs), including high-motility group box-1 (HMGB1), heat shock protein

(HSP), fibrinogen, heparin sulfate, fibronectin, hyaluronic acid, and double- and single-strand RNA.<sup>22</sup> While these molecules are released from necrotic cells, they promote tumor cell survival by activating the TLR4 expressed on tumor cells and subsequently upregulating nuclear factor-kappa B (NF- $\kappa$ B) signaling and antiapoptotic proteins. One study found that the levels of these DAMP-derived molecules were high in the tumor microenvironment and that they induced TLR4-related chronic inflammation, leading to carcinogenesis, cancer progression, and metastasis.<sup>23</sup> Due to alterations in gene expression and the microenvironment (such as hypoxia and malnutrition) of malignant tumors, HMGB1, as well as hyaluronan expression and secretion in tumors and inflammatory cells, are generally enhanced, which plays a potential role in tumorigenesis, growth, infiltration, and metastasis.<sup>24-27</sup> For these reasons, we will further discuss these two endogenous TLR ligands in the context of HCC.

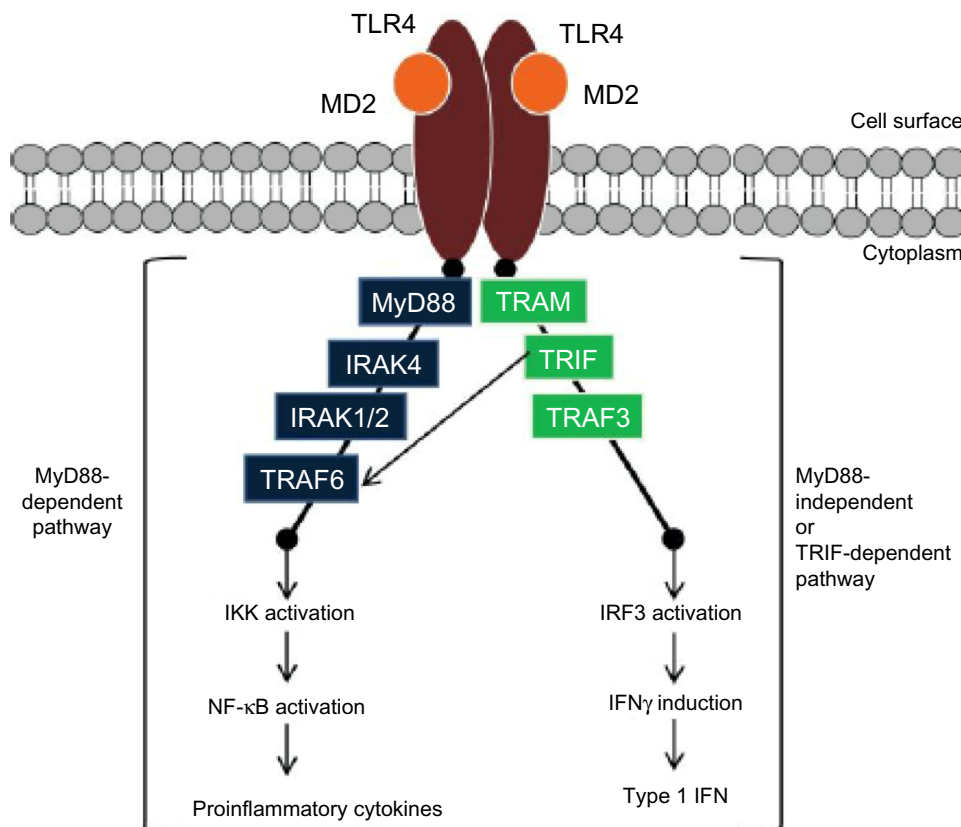
HMGB1 is a DNA-binding protein released from dead cells or secreted by macrophages and hepatocytes at the tumor site that is involved in gene transcription, DNA recombination and repair, and nucleosome remodeling and stabilization.<sup>28</sup> Importantly, extracellular HMGB1 released or secreted by damaged cells has been suggested to be a signature DAMP that indicates the presence of necrosis and subsequently triggers inflammation, cell proliferation, and migration.<sup>29</sup> Recent data suggest that HMGB1 facilitates and amplifies the inflammatory response to different cytokines and TLR agonists.<sup>30,31</sup> Two recent studies found that HMGB1 was highly expressed in HCC tissues and cell lines, induced caspase 1 activation via the TLR4/receptor for advanced glycation endproducts (RAGE) signaling pathway, and mediated the production of various inflammatory cytokines that promote tumor invasion and metastasis.<sup>32,33</sup> Hyaluronan is a negatively charged glycosaminoglycan with a high molecular weight that is ubiquitously distributed in the extracellular matrix. When inflammation or cell damage occurs, hyaluronan can be broken down to fragments via reactive oxygen species, which can then be recognized by TLR4 and leads to the activation of immune cells and the inflammatory response. Hyaluronan is able to stimulate chemokine production in peritoneal macrophages in the absence of CD44 through a TLR2- and TLR4-dependent mechanism.<sup>34</sup> As a major component of the outer membrane in gram-negative bacteria, lipopolysaccharide (LPS) can bind TLR4 and trigger the downstream signaling pathways.<sup>35,36</sup> Ligand-bound TLR4 signals through two pathways. In the myeloid differentiation

factor 88 (MyD88)-dependent pathway, the activated TLR4 recruits the TIR domain-containing adaptor protein and MyD88, which subsequently triggers the downstream adapter proteins interleukin-1 receptor-associated kinase 4, tumor necrosis factor (TNF) receptor-associated factor 6, and transforming growth factor (TGF)- $\beta$ -activated kinase 1, which further activates the I kappa B kinase–NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways, leading to the expression of proinflammatory cytokines. In the MyD88-independent pathway, TLR4 recruits TIR domain-containing adapter-inducing interferon- $\beta$  (TRIF) and the TRIF-related adapter molecule. TRIF indirectly activates transcription factor interferon regulatory factor (IRF)3 and NF- $\kappa$ B, which eventually leads to the transcription of type I interferon and interferon-related genes to contribute to antibacterial and antiviral immunity (Figure 1).<sup>37</sup> TLR stimulation leads to the activation of NF- $\kappa$ B, MAPK, Jun N-terminal kinases, P38, and extracellular signal-regulated kinase, as well as interferon regulatory factor (IRF3, IRF5, and IRF7) signaling pathways, which results in the production of inflammatory cytokines.<sup>38,39</sup> TLR activation in antigen-presenting cells also triggers adaptive immunity.

## TLR4 expression in HCC and hepatocarcinogenesis

In recent years, TLR expression in tumor tissue has been reported, which may provide an important mechanism in the recruitment of inappropriate immune enhancement and dysfunctional immunity and lead to antitumor immune tolerance.<sup>40–43</sup> TLR4 is associated with cancer in several ways. Diverse cell lines and tissue samples derived from patients with head and neck, esophageal, gastric, colorectal, liver, pancreatic, skin, breast, ovarian, and cervical cancer have been shown to express increased amounts of TLR4.<sup>40</sup> In these scenarios of established cancer, TLR4 facilitates an environment that is suitable for continued cancer cell proliferation. Pro-cancer mechanisms could include the evasion of cancer cells from immune surveillance.<sup>14,41–43</sup> The liver may be exposed to bacteria from the intestine via the portal vein, leading to an uncontrolled innate immune system that may result in inflammatory liver disorders.

Although intestinal bacteria maintain a dynamic balance and interact with the host under normal circumstances, the significant increase in *Enterobacter* during chronic liver injury becomes the key pathogenic factor.<sup>44</sup> Many liver



**Figure 1** Schematic illustration of the TLR4 signaling pathway.

**Abbreviations:** TLR, Toll-like receptor; IRAK, interleukin-1 receptor-associated kinase; IKK, inhibitor of nuclear factor-kappa B; IRF, interferon regulatory factor; NF- $\kappa$ B, nuclear factor-kappa B; IFN, interferon.

cells (Kupffer cells, hepatocytes, activated stellate cells) constantly express TLR4.<sup>45–48</sup> Kupffer cells are specialized macrophages located in the liver. Kupffer cells are known to promote hepatic stellate cell (HSC) activation and fibrosis. Fibrosis will eventually cause cirrhosis, or loss of function of the liver.<sup>49,50</sup> Hepatocytes that express TLR4 are responsive to LPS. Hepatocytes are also believed to play a role in the uptake of endotoxin.<sup>51</sup> In response to liver injury, activated HSCs become the major extracellular matrix-producing cell type in the liver. HSCs interact with Kupffer cells and hepatocytes to promote liver fibrosis and inflammatory responses.<sup>52</sup> TLR4 expression was significantly elevated in HBV- and HCV-infected livers,<sup>53,54</sup> as well as in HCC tissues.<sup>55,56</sup> Jing et al<sup>57</sup> detected TLR4 expression in 106 cases of HCC patients' tissue samples; of them, 101 (86%) showed TLR4 positivity. Further studies confirmed that LPS induced the TLR4 signaling that is involved in the invasion and metastasis of HCC and suggested that NF- $\kappa$ B may be directly activated by TLR4 signaling.<sup>57</sup>

We examined TLR4 expression in 60 patients' tumor samples and hepatoma cell lines using reverse transcription polymerase chain reaction assays. One study showed that tumor and normal tissues, as well as HepG2 and H22 cells, expressed TLR4 messenger (m)RNA.<sup>58</sup> Another recent study demonstrated that HCC patients whose tumors expressed high levels of both TLR4 and TLR9 had a poor prognosis.<sup>59</sup> Chronic liver damage caused by excessive inflammation due to the exposure to various risk factors often results in the development of fibrosis-associated HCC.<sup>60</sup> Since stimulation of the TLR4-signaling pathways results in the production of proinflammatory immune mediators, it is likely that TLR4 is involved in the development and progression of hepatocarcinogenesis as well. Seki et al<sup>24</sup> confirmed that LPS/TLR4 signaling promoted liver fibrosis through two independent mechanisms. LPS/TLR4 induced HSCs to secrete chemokines that promote the chemotaxis of Kupffer cells, while TLR4-dependent signaling enhanced TGF- $\beta$  signaling by downregulating bone morphogenetic protein and activin membrane-bound inhibitor.<sup>24</sup> Diethylnitrosamine, a carcinogen, was shown to stimulate TLR4 signaling in mice, resulting in increased tumor size and number, both of which were reduced in MyD88-deficient mice.<sup>61</sup> Dengue-induced apoptotic hepatocytes may activate myeloid cells such as Kupffer cells, induce inflammatory cytokine production and hepatocyte mitogens via the TLR4–MyD88 pathway, and eventually promote HCC development. LPS-induced TLR4 signaling also promotes cancer cell survival and proliferation in HCC.<sup>62,63</sup> HMGB1 was secreted in response to the feeding

second phase of NF- $\kappa$ B activation from HSP70 through TLR4 signaling, which may result in a higher invasion potential of hepatocarcinoma cells.<sup>64</sup> TLR4- and MyD88-deficient mice had lower incidences of HCC and developed significantly fewer and smaller liver tumors compared to the wild-type controls, which implied that TLR4–MyD88 signaling plays a critical role in the development of HCC.<sup>48</sup> Key mediating factors during this process seem to involve TLR–MyD88 signaling and the downstream activation of NF- $\kappa$ B. Collectively, TLR4–MyD88 signaling appears to be essential for hepatocarcinogenesis. Moreover, mutations or polymorphisms of the *TLR4* gene are closely associated with liver carcinogenesis or malignancy.<sup>65</sup>

## TLR4 and the HCC microenvironment

Malignant cells develop intricate mechanisms that enable them to secrete specific cytokines that create an immunosuppressive environment.<sup>3</sup> Studies have correlated elevated TLR expression and dysfunctional immunity within the tumor microenvironment with cancer progression and reduced patient survival in a number of solid tumors.<sup>40,41</sup> TLR signaling in immune cells is critical for the regulation of innate and adaptive immune responses, such as dendritic cell maturation and antigen presentation, as well as CD8+ T-cell cytotoxicity, all of which are important factors in antitumor immunity.<sup>10</sup> TLR activation can also enhance regulatory T-cell suppressor function, favoring tumor development. Tumor-associated macrophages comprise as much as 50% of the tumor mass and provide essential support for the protumor microenvironment.<sup>66</sup> Our study suggested that, in hepatoma cells, TLR4 may indirectly facilitate the recruitment of regulatory T-cells to the tumor site and promote intrahepatic metastasis through its interaction with macrophages.<sup>58</sup>

## Use of TLR4 for HCC therapy

Recent developments in TLR-related applications for cancer therapy have made it an attractive therapeutic target. Many clinical applications using TLR agonists have met with disappointing results when used as monotherapies due to the protumor mechanisms of cancer, which uses TLR expression to facilitate immune suppression.<sup>67–69</sup> TLR agonists have met with greater therapeutic success when used as adjuvants in combination with radiation, chemotherapy, or cancer vaccines by priming the host immunity and leading to an enhanced T-helper (TH) cell cytotoxic or TH1 response. As discussed earlier, chronic inflammation, which plays a critical role in the progression of liver disease and the development of human

cancers, is mediated by TLR activation. Hence, the modulation of TLR4 pathways using various drugs, antibodies, microRNAs (miRNAs), and small molecules that function as TLR4 agonists and antagonists to reduce liver inflammation and prevent the progression of liver diseases toward cancer is a promising strategy to combat HCC.

Lipid A mimetics that bind directly to TLR4–MD2 were shown to inhibit LPS-mediated activation of TLR4 signaling both in vitro and in vivo.<sup>67</sup> A new small molecule, TAK-242, can suppress the expression of inflammatory mediators by selectively inhibiting the TLR4-mediated signaling pathway.<sup>68</sup> Because the intestinal microbiota and TLR4 promote HCC, Schwabe et al<sup>69</sup> found that the nonabsorbable antibiotic rifaximin could moderately reduce hepatocarcinogenesis at late stages, and subsequent TLR4 activation in Kupffer cells has been suggested.

Cowden et al<sup>70</sup> recently tested two inhibitors of the histamine H4 receptor that interact with TLR4 and found that they reduced TNF- $\alpha$  production and LPS-induced inflammation in mouse livers. It has become well established over the past decade that the aberrant expression of a large number of miRNAs correlate with HCC disease severity and poor prognosis.<sup>2,71,72</sup> In bone marrow-derived macrophages and RAW264.7 cells, interleukin-10 inhibits miR-155 expression after LPS stimulation and dampens inflammatory immune responses in a STAT-3-dependent manner.<sup>73</sup> Although the potential of TLR4 immunomodulation for HCC immunotherapy has not yet been extensively explored, initial results from preclinical and clinical studies look promising. It is reasonable to imagine the development of a TLR4 immunomodulatory agent that reduces the inflammatory response but promotes antitumor immunity. This could be beneficial in controlling multiple stages of HCC.

## Conclusion

TLRs play a critical role in innate immunity since they are expressed not only in innate immune cells, but also in nonimmune cells including cancer cells. Functional TLR4 expression has been linked to HCC development. TLR4 may serve an important role in HCC cancer tumorigenesis by promoting the malignant transformation of epithelial cells and tumor growth. The consequences might be dependent on the complex signaling networks triggered by TLR4 activation and the tumor microenvironment. A few studies have suggested TLR4 as a target in the treatment of HCC. Based on these findings, agents targeting TLR4 together with other therapies may be considered the best approaches. Above all, the mechanisms of TLR4 signaling and crosstalk

with other signaling pathways in the HCC microenvironment will definitely provide a promising novel strategy for cancer treatment.

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## Disclosure

The authors report no conflicts of interest in this work.

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