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The Yin and Yang of Toll-like Receptors in Cancer

Jean-Philippe Pradere[1,*], **Dianne H. Dapito**[1,2,*], and **Robert F. Schwabe**[1,2]

[1]Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA

[2]Institute of Human Nutrition, Columbia University, New York, NY 10032, USA

Abstract

Recognition of non-self molecular patterns by pattern recognition receptors is a cornerstone of innate immunity. Toll-like receptors (TLRs) exert a key role in recognizing pathogen-associated molecular patterns (PAMPs) but have also been implicated in the recognition of damage-associated molecular patterns (DAMPs). As such, TLRs regulate a wide range of biological responses including inflammatory and immune responses during carcinogenesis. The high expression of TLRs by antigen-presenting cells, including dendritic cells, and their ability to induce anti-tumor mediators such as type I interferon has led to efforts to utilize TLR agonists in tumor therapy in order to convert the often tolerant immune response towards anti-tumor responses. However, TLRs are also increasingly recognized as regulators of tumor-promoting inflammation and promoters of tumor survival signals. Here, we will review in detail the dichotomous role of TLRs in tumor biology, focusing on relevant TLR-dependent pro- and anti-tumor pathways, and discuss clinical applications of TLR-targeted therapies for tumor prevention and treatment.

Keywords

MyD88; interleukin 6; tumor necrosis factor; NF-κB; Coley's toxin; Imiquimod

Introduction

150 years after Virchow's postulate, the link between inflammation and cancer has been firmly established by epidemiologic investigations and animal studies [1,2]. 18% of human cancers are attributable to infection, and chronic inflammation triggered by infection is an important contributor to carcinogenesis [3,4]. In addition, injury may trigger local inflammation [5], and obesity can contribute to a general hyperinflammatory state [6]. While many tumors arise in the setting of inflammation, it is present in virtually all established tumors. Although inflammation is controlled by numerous mediators and pathways, several systems function as key upstream regulators. Among these are pattern recognition receptors (PRR) that sense danger by detecting non-self or altered molecular patterns. Toll-like

Contact information: Robert F. Schwabe; Department of Medicine, Columbia University, Russ Berrie Pavilion, Room 415, 1150 St. Nicholas Ave, New York, NY 10032; rfs2102@columbia.edu; Tel: (212) 851-5462, Fax: (212) 851-5461.
*These authors contributed equally.

receptors (TLRs) are the best-characterized PRRs for the detection of pathogen-associated molecular patterns (PAMPs). PAMP-induced TLR activation triggers a profound activation of multiple proinflammatory and anti-viral signaling cascades to achieve pathogen elimination. However, if pathogens cannot be eliminated, they may elicit chronic inflammation, which may also in part be mediated through TLRs [7,8]. The important contribution of inflammation to carcinogenesis is best exemplified by high rates of colorectal cancer in inflammatory bowel disease, high rates of gastric cancer in patients infected by *H. pylori*, and high rates of hepatocellular carcinoma in patients with chronic hepatitis [1]. Accordingly, TLR-induced inflammation may act as a promoter of carcinogenesis, with recent studies showing strong promotion of carcinogenesis in the colon, liver, stomach and pancreas by TLRs [9–13]. In addition to PAMP-induced TLR activation, inflammation and injury also induce the release of endogenous molecules termed damage-associated molecular patterns (DAMPs), some of which act as ligands for TLRs [5]. Notably, signals triggered by DAMPs may promote carcinogenesis in a TLR-dependent manner [14]. On the other hand, inflammatory responses may not only provide tumor-promoting signals but also contribute to anti-tumor immune responses [15,16]. After decades of debate, cancer immunosurveillance has been convincingly proven, as demonstrated by increased tumor rates in RAG2−/− mice or mice that lack IFNγ signaling [17]. However, we still do not know how well and in what type of tumors this system works. Based on increased immunogenicity of tumor transplants from immunosuppressed mice, it has been suggested that this system contributes to tumor elimination in early stages, but that escape mechanisms develop, resulting in long-term failure [16]. One interesting aspect linking bacterial PAMPs and anti-tumor responses are historic observations, made by Busch, Bruns and Coley, that bacterial infections such as erysipelas or injection of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* (“Coley’s toxin”) may lead to tumor regression and sometimes even cure [18–21]. Although the mechanisms behind these historic studies remain obscure, recent advances in TLR biology and clinical studies with TLR agonists have suggested that TLR activation may indeed represent a relevant anti-tumor pathway, allowing to convert immune tolerance to anti-tumor immune responses [22–25]. Here, we will briefly review TLR signaling, before discussing the dichotomous role of TLRs in tumor biology, with a particular focus on mechanisms by which TLRs may promote or inhibit cancer. Finally, we will highlight potential applications of TLR-targeting drugs for tumor prevention and therapy.

1. TLRs and TLR signaling

TLR signaling has been reviewed in detail elsewhere [26–28], and we will only summarize key concepts. TLRs are PRRs that recognize a wide range of bacterial, viral and fungal PAMPs, as well as endogenous DAMPs such as high mobility group box 1 (HMGB1). Although individual TLRs recognize distinct ligands, the mechanisms of TLR activation and signal transduction are highly conserved (see Fig. 1). Ligand binding occurs via leucine-rich repeats (LRRs) of extracellular TLR domains and triggers signal transduction pathways through interaction of intracellular Toll/interleukin-1 receptor (TIR) domains with conserved adaptor molecules. Most TLRs signal through the adapter molecule MyD88. Only TLR3 and TLR4 signal through a MyD88-independent pathway that relies on the adapter molecule Trif. TLR4 is the only receptor that signals through MyD88 and Trif. The MyD88-

dependent and MyD88-independent pathways activate multiple proinflammatory signaling cascades including NF-κB, JNK/AP1, ERK and p38, and as well as the interferon pathway [26–28].

2. Tumor promoting actions of TLRs

TLRs may promote carcinogenesis through proinflammatory, anti-apopototic, proliferative and profibrogenic signals in either the tumor microenvironment (TME) or tumor cells themselves, as described below. These effects can be either induced directly in TLR-expressing target cells, or mediated by TLR-induced cytokines.

2.1. TLR-mediated inflammation

TLRs are key regulators of inflammatory signaling, mediated by MyD88-dependent and MyD88-independent pathways. One important tumor-promoting signaling pathway induced by TLR signaling is the transcription factor NF-κB. NF-κB is a master switch of inflammation regulating the transcription of more than 100 proinflammatory genes [29], and is closely related to the avian viral oncoprotein v-Rel [30]. TLR signaling upregulates well-known tumor-promoting inflammatory cytokines through NF-κB-dependent pathways, including IL-1β, TNFα and IL-6 [31–33]. These cytokines promote cancers in the intestine, liver, stomach and skin [34–40]. TLR2-mediated inflammatory signals in macrophages, triggered by tumor-derived, TLR2-agonistic ECM protein versican, promote the secretion of TNFα and metastasis [41]. Besides inflammation, NF-κB activation results in a wide range of cellular responses such as prevention of apoptosis (discussed below), proliferation and anti-oxidant defenses [42,43]. Oxidative stress that typically occurs in the setting of chronic inflammation may not only contribute to the activation of tumor-promoting inflammatory signaling pathways [44] but also change molecular patterns and result in formation of DAMPs with TLR2-activating properties by lipid oxidation [45].

2.2. TLR-mediated anti-apoptotic effects

Resisting cell death is a hallmark of cancer [46,47], counteracting many of the control mechanisms that destroy malignant cells. NF-κB is considered the most relevant anti-apoptotic pathway that controls the expression of anti-apoptotic genes and also restricts the activation of proapoptotic pathways such as JNK [48,49]. TLR signaling activates NF-κB both through MyD88-dependent and MyD88-independent pathways. Moreover, TLRs stimulate the release of cytokines IL-1β and TNFα that in turn promote NF-κB activation in target cells. The key role of TLR signaling in cytoprotection is highlighted by the enhanced susceptibility of MyD88-deficient mice to dextrane sulfate sodium (DSS)-induced colitis [50], which is at least in part mediated by decreased in cytoprotective pathways. Likewise, in the liver and the lung, toxic injury is suppressed in a TLR-dependent manner [9,51,52]. TLR signaling may not only play a role in regulating injury responses in chronically injured precancerous organs but also in promoting survival of malignant cells. As such, TLR-induced NF-κB activation promotes tumor cell survival in colon cancer [53], liver cancer [9], stomach cancer [11] and lung cancer [54]. Moreover, TLR4 on tumor cells mediates resistance of tumor cells to cell death induced by cytotoxic T lymphocytes and promotes tumor growth in vivo [55]. In addition to NF-κB activation in TLR-expressing tumors, survival signals could

also be provided by TLR-expressing cells in the TME such as tumor-associated macrophages (TAM) or cancer-associated fibroblasts (CAF) that release IL-1β or TNFα, which then act on tumor cells (see Fig. 2). Conditional ablation of TLRs in tumor cells and the TME will be required to differentiate between these two possibilities.

2.3. TLR-mediated promotion of wound healing

The similarities between tumor stroma and wounds have led to the suggestion that cancers represent “wounds that do not heal” and a final stage of uncontrolled tissue repair processes [56]. Wound healing responses may strongly promote carcinogenesis as demonstrated by studies with Rous sarcoma virus, in which injury and wound healing of infected animals amplifies carcinogenesis [57]. The two events in the wound healing response that are influenced by TLR signaling include (i) epithelial regeneration and (ii) myofibroblast activation. The liver is the best-known example for the epithelial regeneration with restoration of mass after 70% partial hepatectomy within 7–10 days. Although deletion of single TLRs does not affect regeneration, deletion of the common TLR adapter MyD88 or elimination of intestinally-derived TLR ligands in germ-free mice suppresses liver regeneration [58–60]. A contribution of TLR signaling to regeneration is also found in the intestine, where a TLR2/TLR4/MyD88 signaling cascade mediates epithelial regeneration after DSS-mediated injury [53,61], and in the skin, where lack of MyD88 slows the healing of excisional skin wounds [62]. In the intestine and liver, TLR-MyD88 signaling regulates the expression of mitogenic EGF receptor ligands epiregulin and amphiregulin in non-hematopoietic cells. Accordingly, epiregulin-deficient mice display discreased tumor development in the liver and colon [9,63].

The accumulation of extracellular matrix constitutes a second event that may promote carcinogenesis in a TLR-dependent manner. Fibroblasts and fibroblast precursors functionally express TLR2 [64,65] TLR4 [66–69] and TLR9 [70]. Notably, TLR4 promotes fibrosis development in the liver, as demonstrated by the protective effect of TLR4-, CD14-, MD-2-, MyD88- and Trif-deficiency [71–73]. Mice lacking TLR2 and TLR9 also display a reduction of hepatic fibrosis [70,74]. TLRs exert only moderate profibrogenic effects in other organs, such as skin and kidney, but have no role in lung fibrosis [75].

3. TLR-mediated anti-tumor signals

Although the concept of tumor immunosurveillance is well established, the immune system is much more efficient at triggering dendritic cell (DC) activation in response to pathogens than at recognizing and eliminating tumors. In the past two decades, pharmacologic studies have established that boostering the activation of pathways, which is efficiently triggered by pathogens but not by tumors, such as TLRs and downstream mediators such as type I interferons, can be therapeutically employed to shift the balance from immunotolerance to anti-tumor effects. In particular, TLR7 agonists and interferon alpha have shown therapeutic efficacy in melanoma, basal cell cancer, renal cell cancer, and hairy cell leukemia [76].

3.1. TLR-mediated effects on dendritic cells

Because tumor cells are poor antigen-presenting cells (APC), anti-tumor immune responses typically depend on professional APCs such as DC [77]. As professional APCs, that express a large number of PRR including TLRs and exert powerful effects on lymphocytes, DC are at the interface of innate and adaptive immune responses. Although DC have been in the focus of tumor immunology for their ability to launch potent anti-tumor responses, lacking activation of DC – often a result of inhibitory signals from tumor cells – may also induce immune tolerance through T cell deletion or through regulatory T cells (Treg) [77,78], thus allowing tumor progression. As type I interferon is absolutely required for immune rejection of tumors by DC [79,80], activation of the TLR-IFN signaling pathway represents a therapeutic approach to shift the balance from DC tolerance to anti-tumor response. TLR-activated DC may mediate anti-tumor responses not only through antigen presentation and effects on T cells [81,82] but also through direct cytotoxic effects on tumor cells. Accordingly, both classical and plasmacytoid DC, activated by TLR7 agonists, acquire the ability to induce anti-tumor responses and lyse tumor cells [83–85]. Likewise, TLR5 activation also increases tumoricidal activity of DC [86]. It is possible that DC-mediated tumor killing subsequently triggers amore efficient antigen-presentation to cytotoxic T lymphocytes, thus amplifying anti-tumor responses. Different DC subsets such as TLR9-stimulated plasmacytoid DC and myeloid DC engage in a crosstalk that promotes anti-tumor responses [87].

3.2. TLR- and IFN-mediated effects on natural killer cells

One of the earliest described immunoregulatory functions of type I IFN—its ability to regulate natural killer (NK) cell functions [88] has now been proven to be one of the major mechanisms of the regulation of tumor growth by endogenous type I IFN. Poly I:C-mediated TLR3 stimulation of myeloid DC induces NK activation and NK-mediated tumor regression in a mouse model of melanoma [89]. A novel molecule termed IRF-3-dependent NK-activating molecule (INAM) provides a link between myeloid DC and NK cells[89].

3.3. Inhibition of regulatory T cell functions by TLR signals

$CD4^+CD25^+$ Treg play an important role in immunity and tolerance as evidenced by development of organ-specific autoimmune diseases following their depletion [90]. Activation of TLRs on DC regulates T cell activation not only through MHC class II and costimulatory molecules, but also through TLR-induced signals in DC that block the suppressive effect of Tregs in an IL-6 dependent manner [91]. In addition, synthetic and natural ligands for human TLR8 can directly inhibit Treg cell function, independent of DC, and promote anti-tumor immunity [92]. Altogether, these data demonstrate that TLR activation can release the brake in anti-tumor immunity via Tregs.

3.4. Additional mechanisms through which TLRs exert anti-tumor effects

Treatment with TLR3 agonist poly I:C may induce tumor regression by converting tumor-supporting macrophages to tumor suppressors that produce inflammatory cytokines and promote M1 polarization. This response is mediated by TNFα through a MyD88-independent pathway [93]. TLR9 agonists can also exert anti-tumor effects through suppression of angiogenesis [94]. It is likely that TLR-induced interferons play an important

role as interferon alpha is well known to reduce tumor growth by blocking angiogenesis [95–97].

4. Role of specific TLRs and their ligands in experimental and human carcinogenesis

The most profound contribution of TLRs to carcinogenesis has been observed in organs that are either directly exposed to bacterial TLR ligands such as the GI tract and skin, or indirectly exposed to high amounts of intestinal TLR ligands such as the liver. Thus, tumor-promoting proinflammatory TLR activation seems to be triggered by bacterial PAMPs. Accordingly, germ-free animals display reduced carcinogenesis in the liver and the GI tract [9,98–100]. However, in other organs, e.g. the pancreas, DAMPs may be the main stimulus for TLR-dependent carcinogenesis.

TLRs exert a dichotomous role in cancer. The amplitude and length of receptor activation may have a critical impact on outcome, with chronic low-grade TLR activation favoring a tumor-promoting proinflammatory state, and high-dose therapeutic TLR activation promoting anti-tumor response. Here we summarize results from the most relevant studies and classify TLRs as “largely tumor-promoting” or “largely tumor-suppressing”, with the caveat that TLRs have divergent functions in an organ-, cell-, context- and ligand-dependent manner, and that the literature contains conflicting data.

4.1. TLRs and TLR adapter molecules with largely tumor promoting functions

4.1.1. TLR4—Tumor-promoting functions of TLR4 are best established in the colon, liver, pancreas and skin. In colon, where there is constant interaction between gut microbiota and epithelial cells, TLR4 deletion strongly reduces inflammation and tumor burden in a colitis-associated neoplasia using the azoxymethane (AOM)-DSS model [101]. Furthermore, transgenic mice overexpressing constitutively activated TLR4 in the intestine exhibit a higher sensitivity to colitis-associated neoplasia [102]. These studies are in agreement with other studies in which either deletion of the TLR4 adapter molecule Myd88 [103], or depletion of the gut microbiota, which supplies TLR4-activating PAMPs such as LPS, reduce colon cancer development [103,104]. In contrast, one recent study showed that intestinal overexpression of constitutively activated TLR4 in the APC^{Min} model of colon reduces tumor load by increasing tumor cell apoptosis [105]. In the liver, several studies have demonstrated a tumor-promoting role of TLR4 [9,51]. Moreover, germ-free status or elimination of the gut microbiota by antibiotics decrease hepatocarcinogenesis [9]. Likewise, dysbiosis induced by penicillin increases development of liver cancer whereas treatment with probiotics improves it [106]. TLR4-dependent hepatocarcinogenesis is mediated by non-bone marrow-derived cells. In contrast to these numerous studies, a single study showed increased hepatocarcinogenesis in TLR4-deficient mice [107]. In the skin, TLR4- and Myd88-deficient mice exhibited a strong reduction in tumor numbers induced by two-step chemical carcinogenesis [39,108]. Carcinogenesis in the skin depends on TLR4 expressed by myeloid and epithelial cells, and the endogenous TLR4 ligand HMGB1. MyD88-dependent promotion of skin carcinogenesis not only depends on TLR but also on IL-1R signaling [39]. The fact that TLR4 exerts a protective role in a non-inflammatory model of skin

carcinogenesis suggests that the effect of TLR4 on skin cancer may be context-dependent [109]. In the pancreas, TLR4 deletion decreases tumor growth, while chronic LPS treatment accelerates cancer progression [13]. Tumor-promoting effects of TLR4 in pancreatic cancer are mediated by bone marrow-derived cells [13]. In contrast to largely tumor-promoting role of TLR4 in the colon, liver, skin and pancreas, TLR4 in lung and breast inhibits cancer development: Mice expressing non-functional TLR4 exhibit increased tumor load in a 3-methylcholanthrene-induced lung cancer model [110]. Similarly, TLR4 promotes breast cancer development, progression and metastasis in chemical carcinogenesis and syngeneic transplant models [111,112]. Further studies are required to understand the organ-specific contribution TLR4. It is conceivable that TLR4-activating PAMPs act predominantly as potential tumor promoters, whereas DAMPs function as potential tumor suppressors. Accordingly, chemotherapeutic drugs or radiotherapy require TLR4-activating DAMPs to achieve therapeutic effects: HMGB1 is released following anthracycline treatment or radiotherapy resulting in HMGB1-mediated TLR4 activation on DC and tumor elimination by tumor-specific T cells. [113]. Despite the large body of literature on TLR4 in experimental carcinogenesis, the functional contribution of TLR4 to human carcinogenesis is not well understood. Single nucleotide polymorphisms (SNP) that render TLR4 less responsive, such as Asp299Gly and Thr399Ile, may be associated with precancerous gastric lesions, and increased risk of gallbladder cancer [114,115]. However, large-scale confirmatory studies are missing. The contribution of other TLR4 SNPs to carcinogenesis remains elusive [116]. Patients that received anthracycline-based adjuvant chemotherapy for breast cancer had increased metastasis and decreased metastasis-free survival when carrying the Asp299Gly TLR4 variant, confirming the contribution of TLR4 to chemo- and radiotherapy [113].

4.1.2. TLR2—TLR2 may promote or suppress tumor growth in a context-dependent manner. A profound tumor-promoting role has been observed in gastric cancer. TLR2 is upregulated in human and murine gastric cancer samples [11]. In a genetic mouse model of gastric cancer, deletion of TLR2 results in a drastically reduced tumor load [11]. TLR2-mediated promotion of gastric cancer is independent of bone marrow-derived cells, and is mediated by proliferation and survival signaling pathways including PI3K/Akt, ERK1/2 and NF-κB in the epithelial compartment [11]. In lung cancer, TLR2 exerts tumor- and metastasis-promoting effects. Lung cancer cells secrete extracellular matrix component versican, resulting in versican-mediated TLR2 stimulation and IL-6 and TNF production in macrophage and promotion of metastatic growth [41]. In the liver, TLR2 does not affect hepatocarcinogenesis in the setting of chronic injury [9]. However, in a purely genotoxic model of liver cancer, TLR2 knockout mice exhibit a higher number of tumors and decreased survival [117]. Tumor cells from TLR2 knockout mice showed a lower amount of cell death and a suppressed senescence program. Similar to the liver, the role TLR2 remains controversial in colorectal cancer. In one study, there were no differences in colorectal cancer between wild-type and TLR2-deficient mice using the AOM-DSS model [118]. In contrast, another study reported increased tumor development and higher IL-6, IL-17A and phospho-STAT3 levels in colorectal cancer in TLR2-deficient mice using a similar AOM-DSS model [119]. TLR2 may also be the mediator of angiogenesis-promoting lipid oxidation end products such as ω-(2-carboxyethyl)pyrrole (CEP) [45]. These products are not only

generated during inflammation and wound healing but accumulate in highly vascularized tumors such as melanoma, and induce a VEGF-independent angiogenic response that depends on TLR2 [45]. The role of TLR2 in human carcinogenesis is not well understood. In gastric cancer, STAT3 pathway activation and increased TLR2 expression are associated with poor survival [11]. Although TLR2 polymorphisms have been associated with gastric and cervical cancer risk [120,121], large-scale confirmatory studies are missing.

4.1.3 Adaptor Molecules and Regulators of TLR signaling—Myeloid-differentiation factor 88 (MyD88) mediates downstream signaling of the majority of TLR family members, as well as the receptors for IL-1 and IL-18 (see Fig. 1). The L265P mutation in the TIR domain of MyD88 mutations caused constitutive activation of NF-κB and JAK-STAT pathways and increased survival of malignant cells, thereby contributing to the pathogenesis of diffuse large B-cell lymphoma and Waldenström's macroglobulinemia [122–124]. Conversely, deletion of MyD88 decreases carcinogenesis in many settings, including genetic and chemical models of colon cancer [103], chemically-induced hepatocarcinogenesis [36] and chemical models of skin papilloma and fibrosarcoma [125]. In contrast, deletion of MyD88 results in aggressive fibroinflammation, increased Th2 responses, and profoundly accelerated tumorigenesis in a genetic model of pancreatic cancer [13]. In the latter model, carcinogenesis is driven by the MyD88-independent TRIF pathway, suggesting that MyD88 deletion might promote tumors by overactivation of the TRIF pathway [13]. SIGIRR is a member of the TLR/IL-1 receptor superfamily, and acts as a negative regulator of TLR and IL-1 signaling [126]. Mice deficient in SIGGIR not only display constitutive expression of inflammatory genes in the colon, that depends on the bacterial microbiota, but also increased inflammation following DSS, and increased tumorigenesis in the AOM+DSS model [127].

4.2. TLRs with largely tumor-suppressive functions

4.2.1. TLR3—TLR3 predominantly serves a tumor-suppressive function, largely mediated by TLR3-dependent stimulation of anti-tumor immunity. Accordingly, TLR3 deficiency results in development of acute lymphoblastic T cell leukemia after infection with endogenous retrovirus [128]. In syngeneic and xenograft models of prostate cancer, TLR3 deficient mice display increased tumor growth rate [129]. Conversely, treatment with TLR3 agonist poly I:C inhibits tumor growth in the latter model and a genetic model of prostate cancer overexpressing the TRAMP transgene [129]. Systemic administration of poly I:C reduces the growth of melanoma metastasis [130]. In the liver, there is a positive correlation between TLR3 expression and patient survival [131]. Moreover, poly I:C treatment increased NK cell activation and reduced liver tumor growth in a syngeneic transplant model [131]. Consistently in all models, TLR3 downstream effects are mediated by type I interferon and subsequent NK cell or DC activation, and NK cell- or DC-mediated death of tumor cells. In contrast to the above studies, it has also been suggested that TLR3 could promote tumor development through effects on cell proliferation and survival [132,133].

TLR5: The majority of studies deciphering the contribution of TLR5 to tumor development have revealed potent anti-tumor effects, consistent with the expression and function of TLR5 on DC and their important role in anti-tumor immune responses. These effects are best

exemplified in a study where tumor cell lines expressing TLR5 agonist flagellin fused with a tumor antigen induced DC-mediated anti-tumor immune response [82]. In the colon, knockdown of TLR5 or MyD88 dramatically enhanced growth and inhibited necrosis in a xenograft model [134]. TLR5 is highly expressed in human breast cancer biopsies compared to non-tumor tissue [135]. Implantation of TLR5-deleted breast tumor cells results in enhanced tumor growth while TLR5 agonist flagellin retarded tumor growth by increasing cell death and decreasing tumor cell proliferation [135]. However, the use of immunodeficient mouse models that lack DC, is an important limitation of the above studies. Therefore, the tumor-suppressive function of TLR5 requires additional investigations in mice with a fully functional immune system.

TLR7/TLR8: TLR7 and TLR8 are structurally related receptors for single-stranded RNA, TLR7 and 8 have been the focus of research on TLRs and cancer due to the profound immunomodulatory and anti-tumor effects of small-molecule agonists [136–139]. While there is considerable data on the clinical application of potent TLR7/8 agonists for anti-tumor therapy, relatively little is known about the contribution of endogenous TLR7/8 activation to carcinogenesis. A recent publication demonstrated that microRNAs, released from tumors via exosomes, bind to TLR7 and 8, and favor metastatic growth [140], suggesting that low-level activation of TLR7/8 by endogenous agonists exert tumor-promoting effects. TLR7/8 agonists are predominantly used for anti-tumor therapy of basal cell carcinoma and melanoma [136] (discussed in the next section). Anti-tumor effects are predominantly mediated by TLR7/8 activation on DC, resulting in their recruitment, activation and ability to kill tumor cells [83,85]. Moreover, TLR7/8-activated DC also activate NK cells and suppress Treg [92,141,142]. Despite the overwhelming literature on anti-tumor effects of TLR7/8 agonists recent studies suggest a potential tumor-promoting role TLR7/8 in some settings: In pancreatic cancer, there is not only a high expression of TLR7 in both epithelial and inflammatory cells, but also an acceleration of tumor formation following TLR7 activation [12]. Conversely, pharmacologic inhibition of TLR7 decreases pancreatic cancer. TLR7 exerts its effects on pancreatic cancer in inflammatory cells as chimeric mice with absent TLR7 expression in bone marrow-derived cells displayed decreased carcinogenesis [12]. In the lung, both TLR7 and TLR8 are expressed on tumor cells, and promote the survival of lung cancer cell lines through NF-κB activation and upregulation of Bcl-2 [54]. As discussed above, TLR7/8-agonistic microRNAs promote lung tumor growth [140]. Thus, the effects of TLR7/8 on tumor may depend on the organ, tumor stage, the cell type, and the role that anti-tumor immune responses play in a particular tumor. In light of the potential clinical applications of TLR7/8 agonists for anti-tumor therapies, further pharmacologic and genetic studies are required to understand in which organs and settings TLR7/8 signaling promotes cancer.

TLR9: TLR9 is the receptor for non-methylated CpG DNA that is typically found in viral and bacterial DNA [143]. Similar to TLR7/8, TLR9 has been extensively studied for its role in immunosurveillance and as potential target for anti-tumor therapy using synthetic TLR9 agonists. The contribution of TLR9 to immunosurveillance is highlighted by the fact that TLR9 deficiency results in acute lymphoblastic T cell leukemia after infection with endogenous retrovirus infection [128]. These data suggest that TLR9 may be particularly

relevant for virally induced tumors. Conversely, TLR9 activation by synthetic oligodeoxynucleotide agonists (CpG-ODN) has demonstrated anti-tumor activity in xenograft models of murine cervical carcinoma, neuroblastoma and colon cancer [143–147]. Likewise, the combination of TLR9 agonists with Stat3 siRNA in a single molecule promoted anti-tumor responses against subcutaneously implanted melanoma cells, while at the same time suppressing the Stat3-mediated immunosuppression in the TME [148]. CpG-ODNs are studied for the treatment of different tumor types as a single therapeutic agent [143], as well as in combination with radiation therapy and cytotoxic chemotherapy [149] (discussed in the next section). Mechanistically, TLR9 agonists induce type I interferon secretion in DC resulting in cytotoxic DC, activation of NK cells and cytotoxic T lymphocytes, all of which contribute an antitumor immune response [143,150–153]. In addition to immune cells, TLR9 is also expressed on tumor cells from many tissues [55,154] but the function of TLR9 on tumor cells in vivo is currently not known. As it is conceivable that TLR9 stimulation may be cell- and context-dependent and possibly contribute to tumor cell survival or proliferation, additional studies in TLR9-deficient mice are needed to investigate the functional role of TLR9 in carcinogenesis in vivo [155–158].

5. TLRs as therapeutic targets for tumor prevention and therapy

Once ranked among the top 20 immunotherapy drugs for the cure of cancer, the role of TLRs in cancer biology has not only become more complex with recent studies revealing a tumor-promoting effect of several TLRs, but has also been set back by disappointing results from several phase II and phase III clinical trials [159]. Altogether, these results are leading to more differentiated approaches that requires focus on (i) tumors amenable to immunotherapy, (ii) TLRs that promote anti-tumor responses most efficiently and for which a tumor-promoting role has been excluded, and (iii) combination therapies to boost immune responses to specific antigens, targeting in particular DC. The majority of clinical trials have been performed using TLR3, TLR7/8 and TLR9 agonists (Fig. 3A). On the other side of the spectrum of TLR biology, targeting specific tumor-promoting TLRs such as TLR2 and TLR4, or their ligands for tumor-prevention represents a new and evolving philosophy that could eventually be translated into clinical trials for primary or secondary tumor prevention.

5.1. TLR agonists for the induction of anti-tumor responses

5.1.1. TLR7/8 agonists—The most successful translation of immunomodulatory and anti-cancer effects of all TLR agonists has been achieved for TLR7/8. Accordingly, the TLR7/8 agonist imiquimod is the only TLR agonist approved for cancer therapy. As TLR7 and TLR8 both recognize ssRNA, many synthetic agonists activate both receptors. Several classes of TLR7/8 agonists have been developed [160]: Imidazoquinoline, which resemble adenosine analogues, include the TLR7 agonist imiquimod [138] and the TLR7/8 agonist resiquimod; guanosine analogues such as the TLR7 ligand loxoribine; and ssRNA that has been stabilized to prevent its rapid degradation. Modifications of their RNA sequence can direct ligands specifically towards TLR7 or TLR8 [142]. Among all TLR7/8 ligands, imiquimod is the best studied [136] and in clinical use: Topical administration of Aldara™ (5% imiquimod cream) has been approved by the FDA and the European Union for the treatment of basal cell carcinoma, and achieves a clearance rate of 42–100% [161,162]. Imiquimod is also

being used to locally treat other cutaneous tumors including melanoma with well-documented and >85% success rates for lentigo maligna [163] (Fig. 3B). Evidence is accumulating that it may also be effective in more advanced stages such as invasive cutaneous and locally recurrent melanoma [164–166] Topical imiquimod treatment is also utilized in a phase II clinical trial in breast cancer patients with chest wall recurrence or cutaneous metastasis [22,167] (Fig. 3B). Despite success with topical treatment, the role of TLR7/8 agonists for systemic therapy remains uncertain. Unlike imiquimod, the TLR7 agonist 852A and the TLR8 agonist VTX-2337 can be applied systemically and are currently being evaluated in phase I and phase II clinical trials for various cancers including melanoma breast, ovarian, endometrial, cervical and head and neck cancers (Fig. 3B).

5.1.2. TLR9—Although a large number of clinical trials have been performed using TLR9 agonists, there is currently no FDA-approved TLR9 agonist for tumor therapy. A number of CpG-containing TLR9 agonists have been tested in animal models, including cervical carcinoma, neuroblastoma and colon cancer [143–147], and in clinical trials [22]. Despite promising preclinical studies, several clinical trials have been disappointing, e.g. failure of TLR9 agonists CGP 7909 in a Phase III trial in non-small cell lung cancer, and safety concerns of IMO-2055 in combination with platinum-based therapies Phase II trial in recurrent or metastatic squamous cell carcinoma of the head and neck cancer, respectively [159]. Results from other ongoing studies of TLR9 agonist MGN1703 for metastatic colorectal cancer and TLR9 agonist EMD1201081 for head and neck cancer (Fig. 3B) need to be awaited to judge the role of TLR9 in cancer therapy. Because of these somewhat discouraging clinical studies, focus has shifted towards the ability of TLR9 agonists to activate DC and improve responses to tumor vaccines. The combination of the TLR9 agonist CPG7909, the TLR4 agonist MPL, and the melanoma antigen MAGE-A3 is currently being tested in a Phase III clinical trial (Fig. 3B).

5.1.3. TLR5—Although there is significant data in mice showing anti-tumor effects by TLR5 agonists flagellin [82,135,168] and TLR5-agonistic polyethylenimine-based nanoparticles, that are selectively engulfed by tumor-associated tolerogenic DC [86], translation into clinical practice has not been achieved. CBLB502 (Entolimod) is a TLR5 agonist that is derived from Salmonella flagellin [169] that is currently studied in phase I clinical trials in patients with squamous cell head and neck cancer, and in locally advanced or metastatic solid tumors (Fig. 3B).

5.1.4 TLR3—Despite evidence of tumor-suppressing effects of TLR3 ligand poly I:C in several mouse studies [129,130], there is little data on anti-tumor effects of TLR3 ligands in humans. As poly I:C is inactivated rapidly in humans, several TLR3 ligands have been developed for clinical applications. Poly-ICLC (Hiltinol™) is a derivative of poly I:C that has been stabilized with poly-lysine, and is currently studied in phase II trials for solid tumors (Fig. 3B). Rintatolimod (Ampligen™) is a derivate of poly I:C in which cytidine is replaced by uridine at a ratio of 1:12 (polyI:polyC$_{12}$U). Rintatolimod is employed in combination with vaccines for the treatment of ovarian and fallopian tube cancer, as well as brain tumors (Fig. 3B). The dsRNA mimic IPH-3102 is another TLR3 agonist, but there is no available data on animal studies or clinical trials.

5.1.5 TLR2, TLR4 and mixed agonists—Although TLR2 and TLR4 mediate tumor promotion in many types of mouse cancers, they may exert anti-tumor effects in others, and have been investigated for tumor therapy as well as adjuvants for vaccination. Although strictly not a pure TLR agonist, Bacillus Calmette-Guérin (BCG) mediates many of its immunomodulatory effects through TLR2 and TLR4 [24]. Anti-tumor effects of BCG are known for over 40 years [170–173]. BCG is effective in the treatment of bladder cancer [174–176] (Fig. 3B) and has been approved by the FDA for this purpose. BCG remains the only known effective tumor therapeutic with TLR2/4 agonistic effects. Although BCG has been evaluated in clinical trials for a large number of other tumors, these have been largely disappointing [25]. OM-174 (CXR-526), a derivative from *Escherichia coli* lipid A, is a mixed TLR2/TLR4 agonist. OM-174 has been evaluated in a Phase I study in patients with solid tumors, and is an adjuvant for an autologous DC vaccine for melanoma in a Phase I/II clinical trial (Fig. 3B). Stimuvax is a cancer vaccine against the tumor antigen MUC1 that contains TLR4 agonist monophosphoryl lipid A (MPL), but failed to improve non-small cell lung cancer [177]. In summary, with the exception of the non-specific TLR2/TLR4-agonistic BCG, there is currently no data showing clear anti-tumor activity of TLR2 or TLR4 agonists in clinical trials.

5.2. TLR antagonism for tumor prevention

Based on recent data on tumor promoting effects of TLRs in organs such as the colon, liver and pancreas, inhibition of TLR signaling may represent a novel strategy for tumor prevention. However, knowledge from animal models has not been translated into the clinic and there are currently no ongoing trials. Here we summarize potential therapeutic strategies for tumor prevention:

5.2.1. Modulation of gut microbiome and bacterial translocation by probiotics and antibiotics—The gut microbiome is the main source for bacterial TLR ligands, which may contribute to carcinogenesis in the stomach, colon and liver. Modulating the gut microbiome and/or bacterial translocation by probiotics or antibiotics may represent clinically feasible approaches to reduce TLR-mediated inflammatory and tumor promoting signals. [178]. In murine models of colon cancer, administration of synbiotics but not pro- or prebiotics alone, prevented aberrant crypt foci formation in AOM-induced colon cancer [179,180]. In a rat model of liver carcinogenesis, administration of the probiotic VSL#3 strongly reduced liver tumor formation [106]. In a genetic mouse model of colorectal cancer short-term antibiotic treatment reduces the pro-tumorigenic expression of IL-23 in TAM while long-term depletion of gut microflora, reduces tumor number and size [181]. A recent study confirmed these findings in a different model of colon cancer, where gut sterilization limits the development of colonic dysplasia [182]. In liver cancer, gut sterilization by oral antibiotics results in drastically reduced tumor burden in mouse and rat models [9,51]. Notably, effects of antibiotics are more pronounced when given at late stages of hepatocarcinogenesis, suggesting that gut microbiome modulation by antibiotics might be useful in clinical settings, where early prevention treatment typically is not possible. While the above used antibiotics cannot be applied in patients long-term, recent data also suggests that well tolerated non-absorbable antibiotics such as Rifaximin reduce liver tumors in murine hepatocarcinogenesis [9]. Rifaximin is approved for the treatment of hepatic

encephalopathy and well-tolerated [183]. However, neither probiotics nor antibiotics have yet been studied for primary or secondary tumor prevention in patients. In advanced liver disease, Rifaximin could be used to hit “two birds with one stone” by reducing hepatic encephalopathy and preventing liver cancer development.

5.2.1. TLR2 and TLR4 antagonist for tumor prevention—The TLR4 inhibitors CRX-526 and E5564 are synthetic analogs of the lipid A portion of LPS, and prevent binding of LPS to the TLR4-MD2 complex and TLR4 activation. TAK-242 is a TLR4 antagonist that acts on the intracellular domain of TLR4. Although these TLR antagonists effectively prevent LPS-mediated inflammation and lethality [184–186], they have not been investigated in animal models or clinical trials for tumor prevention. Likewise, the TLR2-inhibitory humanized monoclonal antibody OPN305, reduces inflammation in vivo [187] but has not been investigated for tumor prevention.

Conclusion

Knowledge about the contribution of TLRs to cancer as well as clinical trials employing TLR agonists for anti-tumor therapy have increased tremendously. The most relevant scientific discoveries in the field of TLRs and cancer biology in the past decade has been the realization that TLRs may promote tumor formation in certain organs such as the colon, stomach, pancreas and liver. This knowledge needs to be integrated into current future clinical trials. Although only few approaches have been found to be successful, including the local TLR7 agonist imiquimod and the non-specific TLR2/4 agonist BCG, testing of new TLR agonists, combinations and indications for TLR-based anti-tumor therapy is still ongoing. Integration of TLR agonists as adjuvants into DC-based cancer vaccination has become a new focus [77] that may constitute a new and efficient modality of tumor therapy in the future. Moreover, the tumor-promoting actions of some TLRs may open up new possibilities for primary or secondary tumor prevention. The fact that TLRs may also promote chronic inflammation and cancer in some settings should not deter from successes with TLR agonists as anti-tumor therapy, and requires further mechanistic investigations shedding light on the “Yin and Yang” of TLRs in tumor biology.

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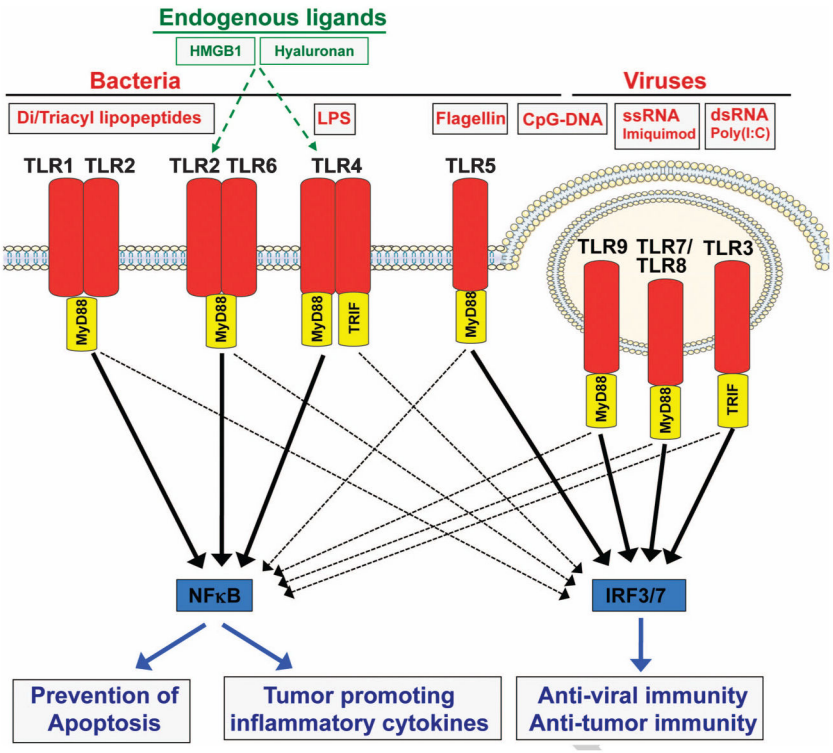


Figure 1. Toll-like receptor signaling

TLRs bind bacterial and viral pathogen-associated molecular patterns (PAMPs), leading to the activation of proinflammatory and anti-viral signaling pathways including NF-κB and IRF3/IRF7, respectively. Activation of these pathways is mediated by two key adaptor molecules MyD88 and Trif. Key biological effects of NF-κB activation include prevention of apoptosis and increased inflammation. Type I interferon induces anti-viral and anti-tumor immunity.

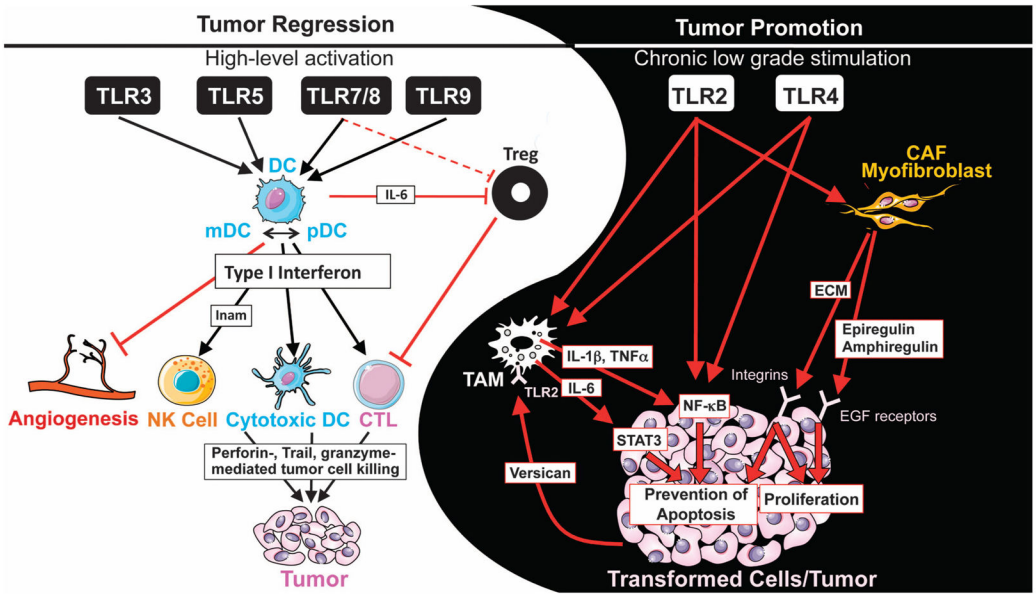
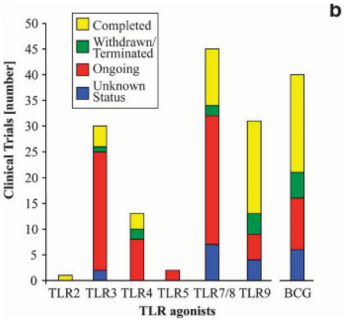


Figure 2. Yin and Yang of TLRs in tumor biology

Potent activation of TLRs, in particular TLR3, TLR5, TLR7/8 and TLR9 may achieve anti-tumor effects by converting immune tolerance into anti-tumor immunity. High level TLR activation induces activation of dendritic cells (DC), which in turn activate key effect cells including natural killer (NK) cells and cytotoxic T lymphocytes (CTL) in a type I interferon-dependent manner. Moreover, DC themselves may also aquire cytotoxic and tumoricidal abilities. In addition, TRL-activated DC also inhibit Treg through IL-6 and angiogenesis through type I interferon. On the other hand, chronic low-grade stimulation of TLRs, in particular TLR2 and TLR4, leads to tumor-promoting inflammation and prevention of tumor apoptosis. This may be mediated through effects on tumor-associated macrophages (TAM), cancer-associated fibroblasts (CAF) or through direct effects in the tumor. NF-κB activation play as key role as it not only fuels inflammation but also prevent apoptosis. Finally, tumor-secreted factors, such as versican, may activate TLRs on cells of the tumor microevironment, further fueling tumor-promoting inflammatory signals.



b

Name	Trade name	Receptor	Cancer	Clinical Phase	Reference
Imiquimod*	Aldara®	TLR7	Basal Cell Carcinoma	FDA approved	Ref. 158,159
BCG*	Theracys®, TICE®	TLR2/4	Bladder Cancer	FDA approved	Ref. 171-173
Imiquimod*	Aldara®	TLR7/8	Melanoma	Phase II	NCT00204555
			Breast cancer (cutaneous, chest wall recurrence)	Phase II	NCT01161888 NCT01421017
852A*		TLR7	Breast, Ovarian	Phase I	NCT00319748
			Ovarian, Cervical	Phase I	NCT00095160
			Solid Tumors	Phase II	NCT00091689
			Melanoma	Phase II	NCT00189332
VTX-2337*		TLR8	Ovarian, Peritoneal Fallopian tube	Phase II	NCT01666444
CPG7909*	Astuprotimut®	TLR9	Melanoma	Phase III	NCT00796445
MGN1703*	dSLIM®	TLR9	Colorectal Cancer	Phase II	NCT01208194
EMD1201081*		TLR9	Head and Neck Cancer	Phase II	NCT01040832
CBLB502*	Entolimod®	TLR5	Head and neck cancer	Phase I	NCT01728480
			Solid Tumors	Phase I	NCT01527136
Poly-ICLC*	Hiltonol®	TLR3	Solid Tumors	Phase II	NCT01734564
Rintatolimod*	Ampligen®	TLR3	Ovarian, Fallopian tube Brain	Phase I/II	NCT01312389
OM-174*		TLR4	Solid Tumors	Phase I	NCT01800812
			Melanoma	Phase I/II	NCT01530698

*TLRs agonists for clinical cancer therapy

Figure 3. TLR agonists for clinical anti-tumor therapies

A. Summary of trials from ClinicalTrial.gov employing TLR agonists or TLR-agonistic Bacillus Calmette-Guérin (BC). **B.** List of FDA-approved TLR agonists for tumor therapy (upper section) and TLR agonists currently being evaluated for tumor therapy (lower section), including the National Clinical Trial (NCT) number from ClinicalTrials.gov.