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Review

The Role of Toll-Like Receptors in Oncotherapy

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Toll-like receptors (TLRs) are associated with tumor growth and immunosuppression, as well as apoptosis and immune system activation. TLRs can activate apoptosis and innate and adaptive immunity pathways, which can be pharmacologically targeted for the development of anticancer oncotherapies. Several studies and clinical trials indicate that TLR agonists are promising adjuvants or elements of novel therapies, particularly when used in conjunction with chemotherapy or radiotherapy. An increasing number of studies suggest that the activation of TLRs in various cancer types is related to oncotherapy; however, before this finding can be applied to clinical practice, additional studies are required. Research suggests that TLR agonists may have potential applications in cancer therapy; nevertheless, because TLR signaling can also promote tumorigenesis, a critical and comprehensive evaluation of TLR action is warranted. This review focuses on recent studies that have assessed the strengths and weaknesses of utilizing TLR agonists as potential anticancer agents.

Key words: Toll-like receptors (TLRs); Oncotherapy; Combination treatment

INTRODUCTION

Toll-like receptors (TLRs) play an important role in innate immunity. These can recognize not only the pathogen-associated molecular patterns (PAMPs) on various microbes but also the damage-associated molecular patterns (DAMPs) from stressed or dying cells[1]. TLR1, 2, 4, 5, 6, and 10 exist in the cell surface, whereas TLR3, 7, 8, and 9 occur in endosomal membranes[2]. TLR11, 12, and 13 are present in mice but not in humans[2]. The activation of TLRs—except for TLR3—leads to the recruitment of the adaptor protein MyD88 to the TIR domain on TLRs[3]. IRAK-4 binds to MyD88, leading to the phosphorylation of IRAK-1, which in turn activates the transcription factor NF-κB[4]. NF-κB translocates to the nucleus, which then regulates the expression of some genes, including proinflammatory cytokine- and survival-related genes[4]. Different TLR signals induced by different cell types could activate MAPKs, JNK, p38, and ERK, leading to the activation of various transcription factors, including AP-1 and CREB[5]. TLR3 transmits signals via the TRIF adapter molecule, and TLR4 recruits TRIF using a bridging adapter TRAM[6]. Therefore, TLR4 is a unique TLR that is able to transmit signals via MyD88 and TRIF. In addition, TRIF can also lead to the activation of NF-κB, ERK, p38, and JNK[7]. The protumor or antitumor effects of the TLR signaling pathway depends on the activation of specific TLRs, cell types, and downstream signaling pathways.

MECHANISMS OF TLR ACTION IN CANCER THERAPY

Research advances involving TLRs show promising applications to cancer therapy. However, understanding the molecular mechanism of TLRs in all types of cancer cells remains the biggest obstacle in clinical therapeutic application. The activation of TLRs not only plays an important role in innate immunity, but it is also linked with the induction of autophagy, apoptosis, or pyroptosis in cancer cells.

The TLR signaling pathway could induce the apoptosis of cancer cells, which activates catabolic enzymes that destroy cellular organelles, ultimately leading to cell death[8]. Studies have suggested that the mechanism of TLR-mediated apoptosis is directly induced by double-stranded RNA (dsRNA) and a TLR3 ligand, and requires the recruitment of receptor-interacting protein 1 (RIP1), caspase 3, and caspase 8. In response to nutrient starvation, autophagy, a tightly regulated mechanism, recycles cellular nutrients to avoid cell death; however, a high level

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of autophagy can eventually lead to cell death. Autophagy is a classical TLR-mediated mode of programmed cell death, but its role in cancer is potentially dichotomous. Research suggests that MAP1S, an autophagy adaptor protein involved in TLR signaling, activates autophagy by regulating Bcl-2/XL and p27 in the noncanonical pathway[9]. Pyroptosis is a proinflammatory reaction that induces programmed cell death and recognizes flagellin components to initiate the process. Researchers have found that flagellin triggers caspase 1-dependent activation of pyroptosis, which is mediated by TLR5 and the neuronal apoptosis inhibitory protein 5 (Naip5)/NLR family CARD domain-containing protein 4 (NLRC4) signaling pathway, to suppress breast cancer cell growth[10]. Additionally, the tumor suppressor p53 also modulates TLR signaling in cancer cells. p53 increases the transcription of TLR genes in response to stress signals and antitumor agents, resulting in enhanced activation of downstream TLR signaling in cancer cells[11]. Moreover, MARVEL domain-containing 1 (MARVELD1), a potential tumor suppressor and a regulator of TLR signaling, can also inhibit the proliferation of cancer cells by regulating p53 and p16[12] (Fig. 1).

TLRs PLAY CONTEXT-DEPENDENT ROLES IN TUMOR PROGRESSION AND METASTASIS

The function of TLR signaling in cancer oncotherapy applications remains controversial. TLR4 has both protective and destructive functions in colitis-associated colorectal cancer[13]. TLR activation leads to cell proliferation in the head and neck and prostate cancers via the NF-κB pathway and is time and lipopolysaccharide (LPS) dose dependent[14]. Research by Paone and coworkers demonstrated that TLR3 stimulation induces apoptosis in prostate cancer[15], suggesting that TLR3 and TLR4 play important roles in cancer development and should be investigated in various tumorigenic settings, including

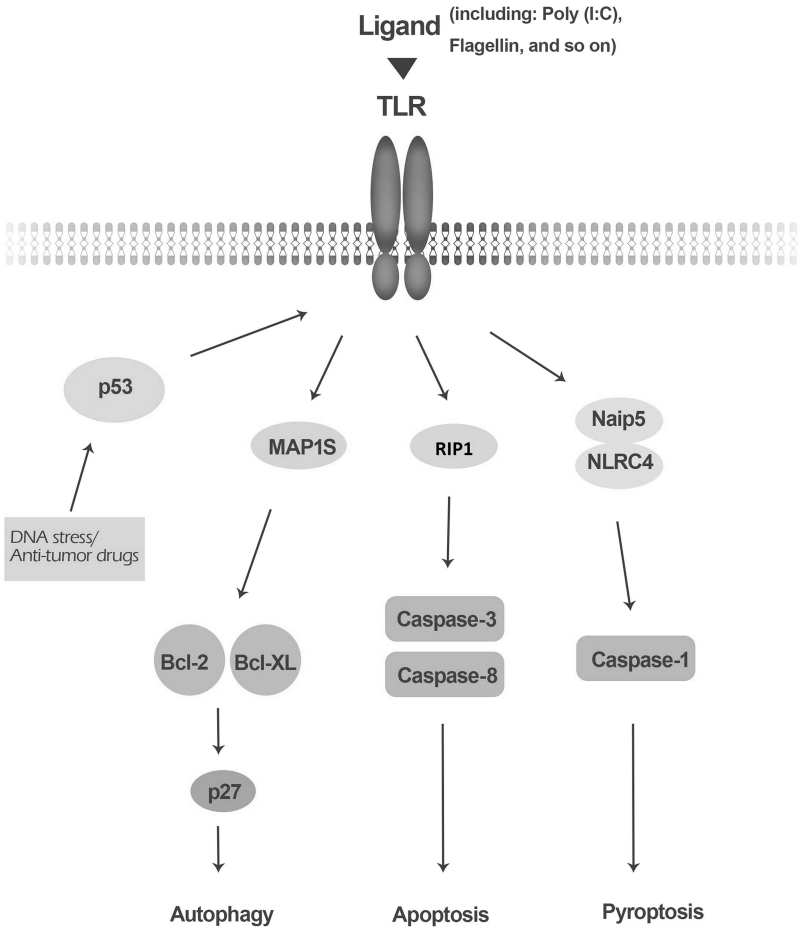

Figure 1. Proposed mechanism of Toll-like receptor (TLR) action for potential application in antitumor therapy. Activation of p53 by DNA stress or anticancer drugs results in enhanced TLR signaling. Activated TLR induces autophagy by modulating Bcl-2/XL and p27 by recruitment of MAP1S through a noncanonical pathway. TLR3 signaling activates caspase 3 and caspase 8 via receptor-interacting protein 1 (RIP1) and induces apoptosis. Flagellin induces cell apoptosis via TLR5 and activates caspase 1 via neuronal apoptosis inhibitory protein 5 (Naip5)/NLR family CARD domain-containing protein 4 (NLRC4) pathway.

colorectal, head and neck, and prostate cancers. TLR4 facilitates tumor immune escape and decreases apoptosis in colon cancer. The activation of TLR4 also induces production of immunosuppressive agents and chemokines, which increase tumor progression and metastasis, including nitric oxide, IL-8, and MMP-9. Additionally, silencing TLR4 in the context of liver cancer has the potential to decrease tumor cell metastasis[16].

In T cells, the expression of TLRs relies on T-cell activation status. Naive T cells express low levels of TLR mRNA, which is increased by T-cell receptor (TCR) signaling[17]. Additionally, TLR effects are dependent on the concomitant stimulation of TCRs. The activation of the TLR and TCR signaling pathways hampers oncotherapy development because of immune suppression and T-cell tolerance, which is induced by regulatory T cells (Tregs) via IL-10 and TGF-β. Moreover, insufficient T-cell stimulation caused by the low affinity of TCRs prevents oncotherapy development[10].

TLRs have different roles in the cancer microenvironment. Poly(I:C) is the most potent type I interferon (IFN) inducer that works via TLR3. In breast cancer, the activation of TLR3 promotes tumor cell death[18]. Specifically, type 1 interferon (IFN) induced by poly(I:C) activates apoptosis in multiple cancer models in mice and humans[19]. Specifically, apoptotic and necrotic cell death are induced by TLR3 stimulation in human colon and lung cancer[20]. Depending on the different stages of cancer progression, TLR3 can also affect cancer cell migration[21]. Importantly, activation of TLR3 inhibits cancer cell migration prior to metastasis (Fig. 2); however, TLR3 activation enhances migration during metastasis[22]. Furthermore, Rutkowski et al. demonstrated that TLR5 increases the responses between IL-6 and tumoral inflammation to boost the mobilization of monocytic myeloid-derived suppressor cells in mice, thereby inducing the growth of tumor cells[23].

When TLRs are activated by pathogens, downstream pathways activate NF-κB, which upregulates the expression of proinflammation cytokines. Hence, inflammatory responses induced by TLRs are related to tumor progression and migration[24]. NF-κB activation leads to the production of IL-1, IL-2, IL-6, and TNF-α, which play immune-suppression roles in the tumor microenvironment[25]. These proinflammatory cytokines promote tumor growth and migration, and the upregulation of cytokines induces the expression of TLRs.

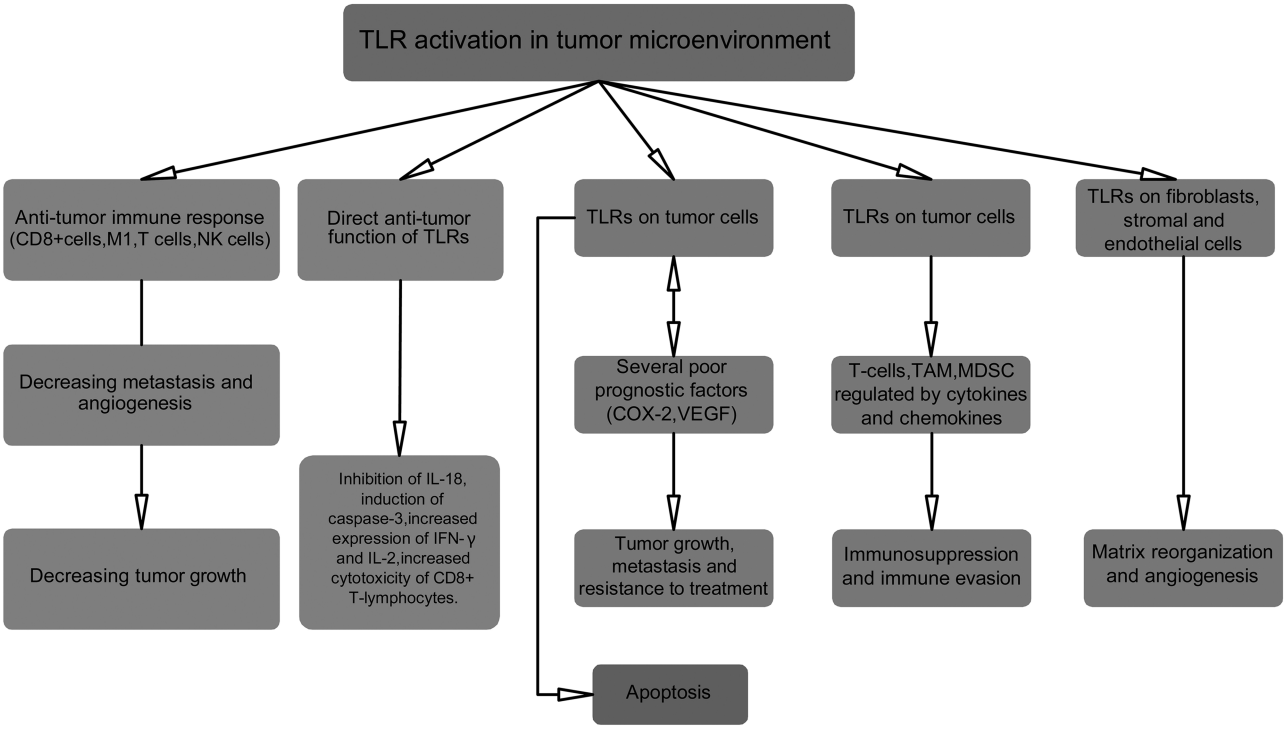

Figure 2. TLR activation in tumorigenesis. In the tumor microenvironment, cancer can lead to chronic inflammation, in turn leading to the activation of TLR. The activation of TLRs leads to immunosuppression and immune evasion of tumor cells. Alternatively, the activation of TLRs activates M1 macrophages, which drive antitumor responses through indirect antitumor activity. TLR activation increases the expression of interferon-α (IFN-α), IL-2, and caspase 3^+ cells and enhances cytotoxic effects of $CD8^+$ T lymphocytes in direct antitumor activity. Overall, activation of TLR signaling presents a kind of balance between the tumor-promoting and antitumor activity.

APPLICATION OF TLR AGONISTS IN CLINICAL TRIALS

Research evaluating antitumor effects of TLR agonists has been conducted for many years, with the majority of the studies focusing on adjuvant properties of TLR agonists in immune systems. Importantly, TLR agonists are most often used in combination with other treatments in the course of cancer therapy. Many clinical trials have focused on evaluating the therapeutic application of TLR ligands and TLR agonists (NCT01800812, NCT02834052, NCT02061449, NCT02180698, NCT 02715882, NCT01861535, NCT02431559); however, only a few of these have published their results (Table 1). Additionally, there is also an ongoing investigation on anti-TLR antibodies (TLR antagonists) as well as inactive analogs of agonists. Although research on TLR agonists has received the most attention for their clinical applications, TLR antagonists are promising agents in the proinflammatory and protumor TLR functions. Additional research into TLR antagonists is required, as most of the antitumor TLR antagonist research studies are still in the preclinical phase.

TLR2 Agonists

Bacillus Calmette–Guerin (BCG), a TLR2/4 agonist, has been approved for the treatment of bladder cancer and has been tested in patients with colorectal cancer in phase 1 clinical trials[26]. OM-174, a TLR2 and TLR4 agonist and a lipid A analog, has proven beneficial in adults with refractory solid tumors during phase 1 trials[17]. OM-174 treatment has proven effective at biologically active concentrations. The combination treatment of OM-174 with chemotherapy showed synergistic effects in animal studies evaluating colon cancer[27]. These results are very promising and this combination therapy should be tested in cancer therapy in patients. Investigations conducted by Jain et al. suggest that SMP105, a TLR2 agonist and a component of the *Mycobacterium bovis* cell wall, increases the number of cytotoxic T-lymphocytes and interferon-producing cells, which in turn reduces cancer growth in mice[28]. However, SMP105 has not yet been utilized in the clinic.

TLR3 Agonists

Poly(I:C), a TLR3 agonist, has been shown to exert antitumor effects in animal models; however, it produced numerous side effects in clinical trials, including hypersensitivity, coagulopathy, renal failure, and shock[29]. The TLR3 TIR domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling pathway can counteract tumor growth by inducing apoptosis, directly promoting the killing of tumor cells and generating a specific memory response for tumor cell-derived antigens in cancer cells[30]. Poly(I:C)12U and poly-ICLC are modifications of poly(I:C), have fewer side effects, and are typically used in combination with standard therapy. In patients with colon cancer, the combination of poly-ICLC with pembrolizumab as an antitumor therapy has been shown to be safe and well tolerated[31]. Poly(I:C)12U has been proven as an effective antitumor treatment for breast, colorectal, and ovarian cancer. Several studies have investigated the effectiveness of the combination treatment of poly-ICLC with radiotherapy or temozolomide in the treatment of glioblastoma, which showed improved effects[32].

TLR4 Agonists

Monophosphoryl lipid A (MPLA) is a TLR4 agonist and a derivative of LPS[23]. The combination of MPLA and alum, known as AS04, is a vaccine that has been utilized in the treatment of various cancers, including metastatic melanoma prostate cancer, and also for the prevention of cervical cancer[33]. MPLA has been investigated in phase 1 clinical trials in patients with colorectal cancer, and it has been approved as an adjuvant used in Cervarix vaccine, which is a prophylactic treatment for human papilloma virus (HPV)-associated cervical cancer[34]. GLA-SE, GSK1795091, and G100 are also TLR4 agonists that are being tested for the treatment of soft tissue sarcoma, Merkel cell carcinoma, and follicular lymphoma[35].

TLR5 Agonists

The TLR5 agonists M-VM3 and CBLB502 are being evaluated for oncotherapy, including prostate cancer, colorectal cancer, and solid and head and neck cancers[36,37]. The results of phase 1 clinical trials using TLR5 agonists are promising, indicating that the therapy was well tolerated and could be coupled with other antitumor therapies, such as chemotherapy or radiotherapy. Moreover, a phase 2 clinical study using CBLB502 is currently ongoing in patients with advanced solid tumors[36]. Additionally, Mett et al. developed an adenovirus-based vector for intratumor delivery, named Mobilan[38]. Mobilan induces the expression of human TLR5 and leads to the secretion of a derivative of *Salmonella* flagellin, which is similar to a TLR5 agonist (entolimod). In cells infected with Mobilan, the expression of TLR5 and its agonist results in an autocrine/paracrine TLR5 signaling loop that can activate NF-κB in vivo and in vitro[38]. Injection of Mobilan into mice with primary prostate tumors results in the upregulation of various inflammatory genes and activation of innate immune cells (e.g., neutrophils and NK cells), which can translocate into the tumors and inhibit tumor progression[38]. Moreover, intratumor injection of Mobilan into the host with subcutaneous prostate tumors improves survival rate after surgical excision owing to decreased tumor metastasis[38].

Table 1. Recent Clinical Trials of Cancer Treatment With Toll-Like Receptor (TLR) Agonists

TLR Agonist	Combination With	Disease	National Clinical Trial Code	State	Phase	Aim
OM-174 (TLR2/TLR4 agonist)	–	Solid tumors	NCT01800812	Completed	I	To determine the maximum tolerated dose, the recommended phase II dose and biological responses associated with OM-174 by intravenous infusion
Poly-ICLC (TLR3 agonist)	Pembrolizumab	Metastatic colon cancer, solid tumor	NCT02834052	Recruiting	I/II	To determine the dose of poly-ICLC that is safe and tolerable when it is combined with pembrolizumab in patients with colon cancer
Poly-ICLC (TLR3 agonist)	Radiation and romidepsin	T-cell lymphoma	NCT02061449	Terminated	I	To evaluate the safety and tolerability of the addition of immunostimulatory therapy consisting of focal radiation with or without the poly ICLC in patients with cutaneous T-cell lymphoma receiving concurrent therapy with the histone deacetylase inhibitor romidepsin
GLA-SE (TLR4 agonist)	Radiotherapy	Stages III and IV adult soft tissue sarcoma	NCT02180698	Active, not recruiting	I	To study phase I clinical trial studies, the side effects, and best dose of GLA-SE when given together with radiotherapy in treating patients with soft tissue sarcoma that has spread to other parts of the body (metastatic) or cannot be removed by surgery (unresectable)
G100 (GLA-SE) (TLR4 agonist)	Pembrolizumab	Follicular low-grade non-Hodgkin's lymphoma	NCT02501473	Recruiting	I/II	To test the safety, immunogenicity, and clinical efficacy of G100 will be examined alone or with pembrolizumab or rituximab
CBLB502 (Entolimod, TLR5 agonist)	Placebo	Colorectal cancer	NCT02715882	Active, not recruiting	II	To study the safety and tolerability of CBLB502 as a neoadjuvant treatment in patients with colorectal cancer, with different doses and regimens
Mobilan (M-VM3) (type V adenovirus carrying TLR5 receptor and its agonist, protein 502s)	Placebo	Prostate cancer	NCT02844699	Active, not recruiting	I/II	To evaluate the safety and efficacy of different regimens of the immunotherapeutic drug, Mobilan, in patients with prostate cancer
Imiquimod (TLR7 agonist)	Surgery	Vulvar intraepithelial neoplasia	NCT01861535	Recruiting	III	To evaluate the efficacy (defined as complete clinical response at 6 months) of imiquimod versus standard treatment (surgery) for vulvar intraepithelial neoplasia

(*continued*)

Table 1. (Continued)

TLR Agonist	Combination With	Disease	National Clinical Trial Code	State	Phase	Aim
Imiquimod (TLR7 agonist)	Paracetamol; lidocaine in Vaseline ointment	Paget disease	NCT02385188	Active, not recruiting	III	To evaluate the efficacy, safety, and immunological response of topical 5% imiquimod cream for noninvasive vulvar Paget's disease
Imiquimod (TLR7 agonist)	Radiotherapy	Lentigo maligna	NCT02394132	Recruiting	III	To investigate the effectiveness of using either radiotherapy or imiquimod to treat the lentigo maligna, when surgery is not possible, is refused, or fails
Motolimod (VTX-2337, TLR8 agonist)	Pegylated liposomal doxorubicin	Ovarian cancer	NCT02431559	Active, not recruiting	I/II	To determine the maximum tolerated dose and the safety profile of the combination of MEDI4736 + motolimod + pegylated liposomal doxorubicin
Motolimod (VTX-2337, TLR8 agonist)	Cyclophosphamide; pegfilgrastim	Metastatic, persistent, recurrent, or progressive solid tumors	NCT02650635	Terminated	I	To study the best way of VTX-2337 and cyclophosphamide in treating patients with a solid tumor that has spread from the primary site to other places in the body (metastatic), progressed for a long time (persistent), come back (recurrent), or is growing, spreading, or getting worse (progressed)
Motolimod (VTX-2337, TLR8 agonist)	Carboplatin; cisplatin; 5-fluorouracil; placebo	Carcinoma, squamous cell of head and neck	NCT01836029	Active, not recruiting	II	To compare the progression-free survival of patients with recurrent or metastatic squamous cell carcinoma of the head and neck treated with VTX-2337 + cisplatin or carboplatin + 5-FU + cetuximab versus patients treated with cisplatin or carboplatin + 5-FU + cetuximab alone
Motolimod (VTX-2337, TLR8 agonist)	Cetuximab; nivolumab	Squamous cell carcinoma of the head and neck	NCT02124850	Recruiting	I	To determine the extent to which the administration of neoadjuvant motolimod plus cetuximab and motolimod plus cetuximab and nivolumab modulates immune biomarkers (NK, mDC, and T-cell activation as well as tumor infiltration and serum cytokines) in peripheral blood and head and neck cancer tumors
EMD 1201081 (TLR9 agonist)	FU; cisplatin; cetuximab	Squamous cell carcinoma of the head and neck	NCT01360827	Terminated	I	To assess the safety and tolerability of EMD 1201081 in combination with 5-FU/cisplatin and cetuximab in first-line treatment of patients with recurrent/metastatic squamous cell carcinoma of the head and neck, and to determine the maximum tolerated dose among the dose levels

TLR7/8 Agonists

The application of TLR7 and TLR8 agonists in oncotherapy has been investigated for several years. Imiquimod (R-837) and resiquimod (R-848) are TLR7 agonists that have been evaluated; however, the results of systemic application of R-837 suggest that it is virulent and may become toxic[39]. Therefore, ongoing research studies on TLR7/8 agonists have focused on the topical application of R-837 and R-848[40]. R-848 can activate TLR7 and TLR8 signaling in mouse models; however, it can only activate TLR8 in humans. Topical application of R-837 was carefully examined in the oncotherapy of various skin tumors such as basal cell cancer[41]. Lentigo maligna is a type of skin cancer (melanomas) involving the head and neck. Traditional therapy requires wide surgical excision; however, this often leads to profound skin defects. Physicians frequently pretreat maligna lesions with 5% imiquimod cream, which may decrease the area of surgical skin defects[42]. Surgery can likely lead to higher rates of reappearance. Therefore, topical therapy with imiquimod could be used as an alternative to surgery for the treatment of lentigo maligna. Safety and efficacy of imiquimod, a TLR7 agonist, were investigated in the treatment of breast cancer with cancer recurrences on the chest wall or cutaneous metastatic carcinoma[43]. The application of 5% imiquimod cream to the wound site for 8 weeks in a study cohort of 10 patients was well tolerated and revealed tumor regression during histological evaluation. Moreover, lymphocytic infiltration of the tumor and local production of cytokines were affected by this treatment, including induction of IFN-α, IL-12, and TNF-α levels, which are linked to immune responses[44]. In contrast, Adams et al. examined the effects of topical therapy with imiquimod cream and surgical excision in nodular basal cell carcinoma, which showed that topical therapy with imiquimod is inferior to surgery[45]. Nevertheless, imiquimod cream can be used as a topical therapy in superficial cancer or nodular basal cell carcinoma[46]. Another study investigated the antitumor effects of 852A, another TLR7 agonist, in the oncotherapy of metastatic ovarian, breast, cervical, and endometrial cancers, which showed no response to standard therapy. However, the findings of that study were limited and confounding as only 3 of the 13 enrolled patients received all the required treatment. Additionally, VTX-2337, a TLR8 agonist, is being investigated in combination with chemotherapy, radiotherapy, and oncotherapy in phase 1 and 2 trials (NCT02650635, NCT01836029, NCT02124850, NCT02431559) in multiple cancers. Klauber et al. developed a targeted delivery system to deliver TLR7 agonist to monocytes, marrow dendritic cells (mDCs), and plasmacytoid dendritic cells (pDCs)[47]. This system can activate target cells and promote the effect of the agonist to trigger the production of antitumor (e.g., IFN-α, IFN-γ, and IL-12p70) and proinflammatory cytokines (e.g., IL-6 and TNF-α). In circulation, targeting leukocyte subsets may deliver activated immune cells to tumor tissues and secrete antitumor cytokines to repolarize the tumor microenvironment. This treatment can be used as an adjuvant therapy after accessible tumor excision and can also be combined with radiotherapy, chemotherapy, or immune inhibitors. Additionally, in leukemia treatment, Villamón et al. found that imiquimod might be a promising treatment option because it imparts a direct cytotoxic effect on leukemic cells and a potential ability to induce differentiation and activate antiacute myelocytic leukemia (AML) cellular immune effects[48].

TLR9 Agonists

The activation of TLR9 induces Th1 type and cytotoxic T-lymphocyte responses. Studies comparing the effects of cetuximab monotherapy and cetuximab combined with EMD 1201081, a TLR9 agonist, in patients with head and neck squamous cell cancer, have revealed that the combination treatment was well tolerated, although the therapeutic effect did not increase[49]. This result suggests that the combination therapy of antigens and TLR agonists may potentially increase the immune response, which has been confirmed in the treatment of melanoma[49]. The combination treatment of AS15 adjuvant, comprising CpG7909 and MAGE-A3 antigen, increased the immune response and improved the therapeutic effect on patients with MAGE-A3^{+} melanoma[50]. Another TLR9 agonist, CpG-ODN, a synthetic oligodeoxynucleotide expressing CpG motifs, can suppress angiogenesis and increase the antitumor effects of chemotherapy and radiotherapy in clinical trials[51,52]. Although TLR9 adjuvants may decrease tumor progression, complete recovery is barely achieved. This may be induced by the activity of suppressor cells, which inhibit tumor-specific immunity in the tumor microenvironment. The safety and efficacy of the TLR9 agonist, CpG7909, were investigated in patients with recurrent low-grade lymphomas who received radiotherapy and CpG7909 vaccination[53,54]. Importantly, in some patients, TLR9 activation induced systemic antilymphoma clinical responses, leading to stable regression of the disease in its entirety. These results suggest that intratumoral vaccination may be effective, and future research should focus on the adjustment of this approach to increase its therapeutic benefits[54].

COMBINED APPLICATION OF TLRs WITH OTHER CANCER TREATMENTS

Radiotherapy is a traditional antitumor treatment that is capable of destroying DNA of exposed tissues and various tumor antigens; however, radiotherapy is unable to achieve the systemic immunosuppression in cancer cells.

The activation of the TLR9 signaling pathway in pDCs and B cells in mouse models using TLR9 agonists imparts an immunostimulatory effect[51]. Therefore, the combination treatment of TLR9 agonist with radiotherapy may be a promising new approach for cancer treatment. In the mouse model of lung cancer, decreasing metastasis of tumor cells and increasing survival rate were achieved by the combination treatment of CpG-ODN with radiotherapy[55]. Moreover, the cotreatment of CBLB502 with radiotherapy was able to alleviate acute radiation symptoms and enhance the efficacy of radiotherapy in killing tumor cells in mouse and monkey models[56]. These results suggest that the TLR combination treatment may generally improve the treatment efficacy and potentially increase survival rates in patients with cancer.

The combination treatment of poly(I:C) or poly-(A:U) with chemotherapy may more effectively suppress tumor cell growth compared to chemotherapy treatment alone[57]. The application of the combination treatment decreases the dosage of administered chemotherapy, thereby reducing the side effects in patients and likely achieving better curative effects[58]. Yoshida et al. reported that poly(I:C) treatment induces the proliferation of antigen-specific cytotoxic T-lymphocytes (CTLs) by antigen-presenting dendritic cells in draining lymph nodes, and the combination treatment of poly(I:C) with radiotherapy leads to an efficient infiltration of CTLs into tumors and the induction of relevant chemokine transcripts[59]. Moreover, poly(I:C)-activated tumor-associated macrophages led to the release of TNF-α, which can act on tumor cells and increase radiotherapy sensitivity. Therefore, the combination treatment of poly(I:C) with radiotherapy may enhance the CTL- and macrophage-dependent antitumor effects to suppress cancer progression.

Oncolytic virus therapy is an effective treatment for cancer. TLR9 activation facilitates oncolytic viruses to induce antitumor immune responses[60]. Studies show that when a repeated CpG island, a TLR9 ligand, is inserted into the genome of an oncolytic virus, a significant increase in viral antitumor effects of this virus is observed[60]. The advantage of the reconstructive oncolytic virus depends on the TLR9 and NK cells, which increases the activation of antigen-specific T cells as well as decreases the activation of myeloid-derived suppressor cells in a mouse model[61].

Spherical and monodisperse gardiquimod-encapsulated poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles (Gardi-PLGA) have the ability to activate TLR7/8[62]. Studies by Seth et al. reported that combination treatment of Gardi-PLGA and 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a vasculature disrupting agent, in bone marrow dendritic cells (BMDCs) and melanoma cells significantly increased the secretion of proinflammatory cytokines in BMDCs, whereas melanoma cells remained viable[62]. Moreover, in a mouse melanoma model, the combination treatment of DMXAA intraperitoneal injection with Gardi-PLGA intratumoral injection significantly suppressed tumor growth and increased survival rate[62]. Additionally, immunohistochemical findings of tumor sections indicated vasculature disruption following treatment with DMXAA in vivo. These results suggest that the synergistic effect of immune stimulation caused by DC activation and vasculature disruption influenced the inhibition of tumor growth.

Müller et al. found that macrophages were not able to destroy tumor cells by treatment with IFN-γ or TLR agonists alone[63]. However, induction of macrophage tumoricidal effect and production of NO and proinflammatory cytokines (TNF-α, IL12p40, and IL-12p70) were induced by combination treatment of IFN-γ with TLR agonists [TLR1/2 agonist Pam3, TLR2/6 agonist staphylococcal lipoteichoic acid (LTA), TLR3 agonist poly(I:C), TLR5 agonist flagellin, TLR7 agonist CL264, and TLR9 agonist CpG][63]. Studies suggest that combined signals of IFN-γ and TLR agonists from tumor microenvironment were necessary for optimal induction of antitumor M1 macrophages, and combination treatment of IFN-γ with TLR agonists may provide a new strategy for cancer oncotherapy.

Kurkjian et al. reported that the synthetic TLR2/6 agonist, fibroblast-stimulating lipopeptide (FSL-1), prolonged survival in male and female mice after 24 h of radiation[64]. FSL-1 has been shown to not only promote hematopoiesis in bone marrow, spleen, and periphery but also to increase systemic levels of hematopoiesis-stimulating factors[65]. Radiation leads to hematopoietic dysfunction; therefore, induction of hematopoiesis with FSL-1 may play an important role. Hence, the combination treatment of FSL-1 with radiotherapy may reduce side effects caused by radiotherapy alone when used in cancer treatment.

TLRs impart immune inhibitory effects through the production of various immunosuppressive factors, such as Tregs, PD-L1, and IL-10, which could inhibit antitumor immunity[66]. Some TLRs are able to induce Tregs or PD-L1 to promote cancer progression, such as TLR4. Resiquimod and LPS induce PD-L1 on DCs and then lead to the development of tolerogenic antigen-presenting cells (APC)[67]. Activation of TLR4 induced by LPS leads to PD-L1 synthesis in tumor cells, thereby inducing resistance between the tumor cells and CTL attack[68]. Besides, induction of PD-L1 plays an important role in the restriction of antitumor effects of TLR agonists in mouse models[66,69]. Poly(I:C) upregulates the expression of PD-L1 on DCs, and consumption or blockade of PD-L1 increases the cell expansion of $CD8^+$ T cells on activated DCs[66]. Combination treatment with TLR agonists and PD-L1 blockade leads to complete tumor regression and

establishes long-acting protective immunity[69]. Therefore, blocking PD-L1 may enhance the antitumor effects of TLR agonists. A phase I/II study involving VTX-2337 and MEDI4736 (anti-PD-L1 antibody) in subjects with recurrent, platinum-resistant ovarian cancer for whom PEGylated liposomal doxorubicin (PLD) is currently testing the effects of combination treatment with TLR agonist and PD-L1 blockade (NCT02431559). Epstein–Barr virus (EBV) increases the expression of PD-L1 through the TLR signaling pathway, which may be an immune-evasion mechanism induced by viruses. This strategy may result in dysregulated immune responses of some diseases related to EBV, including T-cell lymphoma and nasopharyngeal cancer[70].

MULTIPLE AGONIST TREATMENT IN CANCER ONCOTHERAPY

US Food and Drug Administration (US FDA) approved several TLR agonists for the treatment of cancer patients, including BCG (TLR2/3/4 and possibly TLR9 agonist), MPLA (TLR4 agonist), and imiquimod (TLR7 agonist)[1]. BCG treatment has the most promising antitumor effects in the bladder cancer patients, where suppressing both TLR2 and TLR4 blocks BCG-mediated DC maturation by 70% and suppression of either TLR2 or TLR4 individually inhibits the process by 30% to 40%. Moreover, the activation of TLR2 and TLR4 leads to very strong immunostimulatory BCG effects, which highlights the superiority of activation of multiple TLRs in cancer therapy[71]. BCG is the only FDA-approved therapy for bladder cancer treatment and is currently being tested for other types of cancers in clinical trials. Ongoing research efforts are focused on improving the specificity and efficacy of the antitumor effects of BCG treatment. Specifically, the combination treatment of BCG with autologous whole cell tumor lysate vaccines shows increasing antitumor effects and enhanced survival rate in patients with colorectal, melanoma, and renal cell cancers[72]. Although intense T-cell responses may be induced by this vaccination, we cannot exclude the role of antibody and innate immune cell responses. Melanoma patients who did not receive successive vaccinations with BCG exhibited decreasing delayed-type hypersensitivity (DTH) reactions associated with the antitumor responses. These results suggest that the antitumor responses require a continuous TLR agonist source.

Adding tumor lysates into vaccines may stimulate additional TLRs and/or other pattern recognition receptors (PRRs) to achieve a better effect through lysate-derived DAMPs[73]. The stimulation of TLRs on dendritic cells (DCs) plays an important role in the induction of tumor-specific T cells; however, an additional investigation into antitumor responses of patients as a result of TLR stimulation on T cells or tumor cells is required.

Caisová et al. developed a combination treatment consisting of mannan anchored to tumor cell surface by a biocompatible membrane anchor, two TLR agonists [resiquimod and poly(I:C)], and lipoteichoic acid[74]. This treatment led to 83% reduction in progressive melanoma in a mouse model and led to resistance of tumor cell metastasis. Hotz et al. developed a sequential combination treatment of TLR7 agonist with poly(I:C), which was capable of activating NK cells and cytotoxic T-cell responses with additional advantages for oncotherapy, including reduction of cancer progression[75]. TLR7 stimulation has been shown to exhibit tumor-promoting effects likely owing to the TLR expression by tumor cells, leading to enhanced tumor growth and resistance to chemotherapy. However, proapoptosis and autophagy induced by poly(I:C) may counteract the tumor-promoting effects of TLR7 agonists; therefore, this sequential therapy may offer pure antitumor effects mediated by immune cells. Bocanegra Gondan et al. reported a vaccine consisting of TLR agonists, poly(I:C) and R837 (TLR7 agonist), model antigen ovalbumin (OVA), and phospholipid micelles loaded with zinc-doped iron oxide magnetic nanoparticles[76]. Treatment with this vaccine exhibited an obvious effect against B16-F10 melanoma cells by inducing an intense innate immune response in the lymph nodes without causing a systemic release of proinflammatory cytokines[76]. These results suggest that combination vaccine with synergistic TLR agonists and magnetic nanoparticles together with immune checkpoint blockade has promising applications in the clinical development of vaccines for cancer oncotherapy.

Kuai et al. reported the use of homogeneous and ultrasmall nanodiscs, which can carry multiple adjuvants, including TL4 agonist MPLA and TLR9 agonist CpG[77]. Nanodiscs with these two adjuvants increased costimulatory signals, leading to the induction of proinflammatory cytokines and effective activation of DCs compared to nanodiscs with only one adjuvant[77]. Moreover, studies indicate that the adjuvant-loaded nanodiscs exert potent antitumor effects after vaccination with tumor antigens and lead to the regression of tumors in multiple mouse models.

Recent studies have demonstrated the advantages of TLR synergy in preclinical studies[78]. Ahonen et al. found that injection of poly(A:U) or CpG-ODN alone did not reduce tumor growth, while the injection of the two agonists and tumor-associated antigen was effective in controlling the growth of tumor cells in mice[79]. Zhu and coworkers demonstrated that using specific two types of TLR agonists led to a synergistic effect in activating DCs and promoting antigen-specific T-cell responses and proliferation when compared to using a single TLR agonist[80]. Zhu et al. also reported that the suboptimal doses of combination treatment of TLR2, 3, and TLR9 ligands exerted

a synergistic effect, which were dependent on the crosstalk between MyD88 and TRIF signaling pathway[81]. Bayyurt et al. reported that liposomes coencapsulating CpG-ODN and poly(I:C) promoted cellular uptake and improved the production of proinflammatory cytokines together with secretion of type 1/2 IFN, leading to increased APC function and antigen processing[82]. The coencapsulation of OVA antigen into liposome vesicles displayed a persistent antitumor effect[83]. In addition, enhanced Th1 immunity not only led to the induction of OVA-specific memory $CD8^+$ T cells but also suppressed tumor growth. Conforti et al. demonstrated that combination treatment of imiquimod with CD40 agonists displayed a synergistic effect and increased $CD8^+$ T-cell responses about 20-fold compared to a single agonist[84].

The expression of cytokine and costimulatory molecules on APCs or tumor cells activated by TLR agonists determines the pro- or antitumor response. The ideal TLR mixture would consist of the agonist capable of maturing DCs and producing cytokines that increase T-cell activation while exerting cytotoxic or cytostatic effects on tumor cells[85]. The activation of TLR9 triggers the production of IL-12 and IFN-γ by the DCs, whereas the activation of TLR1 and TLR2 on CD8 and CD4 T cells promotes survival, proliferation, and cytotoxicity of tumor cells[86]. Activation of several TLRs on human and mouse DCs has been shown to increase production of the inflammatory IL-12 mediator, which can skew the immunity toward the antitumor Th1 response. However, the generation of the Th2 response, which depends on TLR agonists, can also be induced by the MyD88-dependent TLR signaling pathway. Although the activation of TLR4 alone induces the production of DCs, which express IL-4 (Th2 type), the activation of TLR7/8 and TLR4 tends to produce the DCs expressing IFN-γ (Th1 type)[87]. Therefore, additional studies are needed to verify whether a combination treatment of TLR agonists can increase the production of cytokines that are able to support the protumor environment via the CD4 Tregs or other immunosuppressive mechanisms. Additionally, DC maturation mediated by TLRs must be capable of inducing the expression of costimulatory molecules for T-cell activation. Hong et al. reported that the activation of MyD88 and TRIF induced expression of IL-6 and IL-12p70 and increased the expression of CD40, CD70, and CD86 in DCs[88]. Under these conditions, stimulated T cells became resistant to the suppressive effects of CD4 Tregs.

However, some matters require consideration. Specifically, the combination treatment of TLR9 agonist EMD 1201081 with platinum-based therapies has been stopped for the treatment of metastatic squamous cell carcinoma based on potential safety concerns (NCT01360827). In another study, TLR9 agonist IMO 2055 did not improve survival rate in the phase 2 clinical trials in the recurrent or metastatic head and neck tumors[89]. Finally, the combination treatment of TLR9 agonist CpG7909 with chemotherapy has also been terminated in phase 3 clinical trials for non-small cell lung cancer[90].

CONCLUSIONS

Studies indicate that TLR agonists can induce innate and adaptive immune responses. For these reasons, TLR agonists are the potential targets for the development of cancer therapies. The results of recent studies indicate that the best application of TLR agonists in oncotherapy is the combination of TLR agonists with other therapy methods, including TLR agonists, monoclonal antibodies, cancer-associated antigens, siRNA, and conventional therapies, including chemotherapy, radiotherapy, or surgery. New delivery systems, including nanomaterials that can produce nanovaccines or deliver drugs/genes/proteins to designated location, should also require further investigations. The advantages of combination therapy include lower dosage of the drug and lower side effects, as well as a potential decrease in therapeutic resistance or activation of immune response. Therefore, the combination treatment is likely to be more effective and provide better antitumor effects. However, many studies still report that TLR ligands have protumor effects in various cancers, and increasing expression of TLRs in different tumor tissues is related to poor prognosis, recurrence, and low survival rate in cancer patients. Additionally, some studies demonstrate that the existence of endogenous TLR ligands may be derived from cell death induced by chemotherapy and can promote immune evasion or cancer growth. In summary, additional in vitro and in vivo studies are needed to assess the role of TLR ligands in cancer progression and cancer prevention.

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