Chapter X

TLR Agonists as Adjuvants for Cancer Vaccines

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Abstract

Toll-like receptors (TLRs) are one of the best characterised family of pattern recognition receptors (PRRs) and play a critical role in the host defence to infection. Accumulating evidence indicates that TLRs also participate in maintaining tissue homeostasis by controlling inflammation and tissue repair, as well as promoting anti-tumor effects via activation and modulation of adaptive immune responses. TLR agonists have successfully been exploited to ameliorate the efficacy of various cancer therapies. In this chapter, we will discuss the rationales of using TLR agonists as adjuvants to cancer treatments and summarise the recent findings of preclinical and clinical studies of TLR agonism-based cancer therapies.

X.1 Toll-like receptors

TLRs are a class of single membrane-spanning, catalytically-inactive receptors. 10 human, and 13 mouse TLRs have been classified to date [1, 2]. This protein family are best known for their ability to detect conserved microbial components, so called PAMPs (pathogen-associated molecular patterns) [3]. The well characterised TLR microbial ligands include: bacterial lipopolysaccharide (LPS) and its derivatives which activate TLR4; lipotechoic acid and lipoprotein from bacterial cell wall and fugal zymosan which stimulate TLR1, TLR2 and TLR6; bacterial flagellin which is sensed by TLR5. Additionally, unmethylated bacterial DNA stimulates TLR9; double-stranded RNA activates TLR3; and single-stranded RNA (ssRNA) is recognised by both TLR7 and TLR8. The cellular localisation of TLRs largely reflect their function and mode of ligand interaction. For example, the TLRs that recognise viral and bacterial nucleic acids such as TLR3, TLR7, TLR8 and TLR9 are mainly localised

endosomally, while TLRs on the cell surface such as TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are involved in detection of bacterial components in the extracellular space. Several TLRs, particularly TLR2 and TLR4 have been shown to detect not only exogenous PAMPs but also host derived endogenous "damage associated molecular patterns" (DAMPs) [4]. Many such ligands are increasingly being identified and include heat shock proteins, high mobility group box1 (HMGB1), various metabolic products such as reactive oxygen species (ROS) and uric acid as well as extracellular matrix components such as fibronectin and hyaluronan fragments [4].

Early studies suggested that TLRs are preferentially expressed on innate immune cells where the types of TLRs and level of expression are governed by cell-type specificity and function, which is associated with specific cytokine production [5-8]. More recent data demonstrates that TLRs are also expressed on epithelial cells of the gastrointestinal, urogenital and respiratory tracts where they play important roles in the first line defence against infection. Additionally, they may also function to preserve epithelial barrier integrity [9-11]. Ligand binding of TLRs induces dimerization and conformational change which activates two major signaling cascades – the MyD88-dependent or -independent pathways. Ultimately, this results in the activation and transduction of numerous downstream pathways including nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs) and interferon regulatory factors (IRFs) to induce the upregulation of type I interferons (IFNs), and various pro-inflammatory cytokines (e.g. Type I IFN, IL-1, IL-6, TNFα etc.) and chemokines (e.g. MCP-1, CCL and CXCL chemokines). Given the canonical outcomes of TLR activation, the past decade has seen considerable effort in ways to engage or modulate TLR signaling as potential therapeutic targets capable of enhancing anti-tumor effect by orchestrating innate responses and activation of the adaptive immune system [12, 13]. The basic immunology of TLR recognition of ligands and mechanisms of signal transduction have been extensively reviewed elsewhere. Rather, this chapter focuses on TLR agonists as adjuvants for cancer immunotherapy in preclinical and clinical studies.

X.2 Cancer vaccine and adjuvant

Cancer vaccines, being prophylactic or therapeutic, aim to stimulate or restore the immune system's capacity to protect against persistent infection that initiate and drive oncogenesis. Prophylactic vaccination against human papillomavirus–induced genital cancers with Gardasil® or Cervarix® vaccines, or hepatitis B–induced hepatic cancers with Engerix®-B or Recombivax HB® vaccines have been used worldwide and play an important role in public health [14, 15]. Despite several decades of intense research and clinical evaluation, only one therapeutic vaccine (Provenge®) has been approved by FDA for the treatment cancer - androgen-independent metastatic prostate cancer [16]. In addition to drug safety profiles, there remain a few challenges that impact on vaccine efficacy that must be overcome. For example, the major drawbacks for currently used purified tumor associated antigens (TAA) such as DNA/RNA, recombinant protein and peptides have poor immunogenicity and may cause inappropriate immune response that elicit no benefit or protection against the targeted infection or malignancy [17-19]. To overcome this and to be more effective, adjuvants are often co-administrated within the vaccine. In most cases, adjuvants were designed to augment the magnitude of an adaptive response to the vaccine administered.

Based on their perceived mechanism of action, adjuvants in the clinical settings can be divided into two main classes: immunostimulators and delivery system adjuvants [18]. Many natural and synthetic agents can be used as adjuvants such as emulsions of liposomes or PAMPs [18]. A carefully designed formulation of adjuvant-vaccine combination is required for directing appropriate types of responses and for achieving synergism, which is otherwise difficult to achieve with a single adjuvant. For example, Cervarix, the prophylactic cancer vaccine against various types of human papilloma virus (HPV) contain purified virus-like particles (VLPs) of the major capsid (L1) protein of HPV types 6, 11, 16 and 18. Purified VLPs are then absorbed on aluminium hydroxy sulphate particles which act as the first adjuvant, the delivery system as aluminium typically induces Th2 immune profiling. Monophosphoryl lipid A (MPL), the TLR-4 agonist, is then added as the second adjuvant which broadens the immune responses [20]. Substantial evidence showed that using natural ligands or synthetic agonists for PRRs as adjuvants, can activate multiple elements of innate immunity. A number of TLR agonists are now in clinical or preclinical studies either standalone or with many different combinations for improving therapeutic cancer vaccines. So far, three TLR agonists have been licensed

by the FDA for use in human cancers, including: BCG (the bacillus Calmette-Guerin) - a mixed TLR2/TLR4 agonist originally developed as an anti-tuberculosis vaccine but is currently approved for *in situ* bladder cancer vaccine; MPL (monophosphoryl lipid A) - also a mixed TLR2/TLR4 agonist derived from LPS that is used as an immunostimulatory adjuvant as part of Cervarix and finally Imiquimod - an imidazoquinoline derivative that can function via TLR7 dependent or independent mechanisms [21] that have been tested in many human malignancies.

Table 1. The representative TLR agonists used in clinical trials for cancer therapy

Agent	TLR	Compound source	Indication	Notes	Reference
852A	TLR7	A synthetic imidazoquinoline	Leukemia, lymphoma, melanoma	Sustained tolerable using prolonged s.c schedule [22]	NCT00276159 , NCT00319748 , NCT00091689
Ampligen (rintatolimod)	TLR3	polyIC12U	Breast Ca, Ovarian Ca, Colorectal Ca	Th1 response [23]	NCT01355393, NCT01312389, NCT01545141
BCG	TLR2,4	*Mycobacterium bovis*	Bladder Ca	Intravesical use, FDA approved [24]	NCT02365207, NCT02015104
Cadi-05	polyTLR agonist	*Mycobacterium indicus pranii*	Melanoma	IFNγ☐ dependent anti-tumor effect [25]	NCT00675727
CBLB502 (Entolimod)	TLR5	*Salmonella enterica flagellin*	Colorectal Ca, SCHNC	Radioprotective and organ specific immnuadjuvants [26]	NCT02715882, NCT01728480
CpG7909 (Promune)	TLR9	class B unmethylated CpG oligonucleotides	Renal cell Ca, NSCLC, Breast Ca	Stat1 dependent cell death [25]	NCT00043407, NCT00070629, NCT00043394
DIMS0150 (Kappaproct)	TLR9	ssDNA-based synthetic ODN	UC	enhance the steroid sensitivity [27]	NCT01493960
Hiltonol	TLR3 MDA-5	polyICLC	Non-melanoma skin cancer, glioma, lymphoma	Stable and heat tolerant [19]	NCT02423863, NCT01188096, NCT01976585
Imiquimod	TLR7	imidazoquinoline	Basal cell Ca, CIN	FDA approved [17]	NCT01264731, NCT02917746, NCT02917746
IMM-101	polyTLR agonist	*Mycobacterium obuense*	Melonoma, unresectable Ca, pancreas Ca	Restore Th1 and downregulate Th2 [28]	NCT01559818, NCT03009058, NCT01303172
IMO-2055 (EMD1201081)	TLR9	phosphorothioate oligodeoxynucleotide	Renal cell Ca, NSCLC	Impair EGFR signaling [29]	NCT00729053, NCT00633529
ISS 1018	TLR9	phosphorothioate oligodeoxynucleotide	lymphoma	Synergic effects with Rituximab [30]	NCT00251394

OM-174 (CRX-527)	TLR2,4	Escherichia coli.	Solid tumors	Well tolerated in phase I [31]	NCT01800812
Picibanil (OK-432)	TLR2,4	*streptococcus.pyogenes*	Pancreas Ca, AEC	DC maturation [32]	NCT00795977, NCT00291473
Resiquimod	TLR7, TLR8	imidazoquinoline	Various cancer type	Testing in numerous combination therapy [17]	NCT00821652, NCT00470379, NCT01676831
SD-101	TLR9	phosphorothiolate ODN	Lymphoma, SCCHN	resensitize to PD-1 blockade [33]	NCT02521870, NCT02927964
TMX-101	TLR7	Imiquimod	Bladder Ca *in situ*	IL-6 and IL-18 induction [34]	NCT01731652
VTX-2337 (Motolimod)	TLR8	A small-molecule agonist	Ovarian Ca, SCCHN	Strong IL-12 and TNFα production [35]	NCT02431559, NCT02124850

Abbreviation: pDC: plasmacytoid dendritic cell; s.c: Subcutaneously; BCG: the bacillus Calmette-Guerin; polyICLC: particular formulation of polyI:C that includes carboxymethylcellulose and poly-L-lysine as stabilizing agents. NSCLC: non-small cell lung cancer. ODN: oligodeoxyribonucleotide. SCHNC: Squamous Cell Head and Neck Cancer. UC: ulcerative colitis. CIN: Cervical Intraepithelial Neoplasia. AEC: Antigen-Expressing Cancers

X.3 TLR agonists as adjuvants

A rational for adjuvanting vaccines is to induce the synchronous activation of dendritic cell (DC) presenting antigen and promote Th1 and CD8+ T cell responses with minimal adverse effects. Extensive mouse studies and human trials using synthetic TLR agonists have demonstrated that adding TLR agonists to cancer treatments profoundly influence the extent of adaptive immune responses to tumor antigens. Such enhancing effects include direct activation of DC subsets, type I IFN production, enhanced cross presentation, augmented CD8+ T cell responses and increased antibody titers; reinvigorated immunesurveilance in patients whose immune system are compromised or in less immunogenic patients such as elderly and children (change the tumor environment); sensitized conventional chemotherapy or radiotherapy; dose sparing, either minimizing the number or amount of antigen introduced or reducing the vaccine schedule for optimal effects [20, 36]. The key effects of TLR agonists on immune system are summarised in Figure 1.
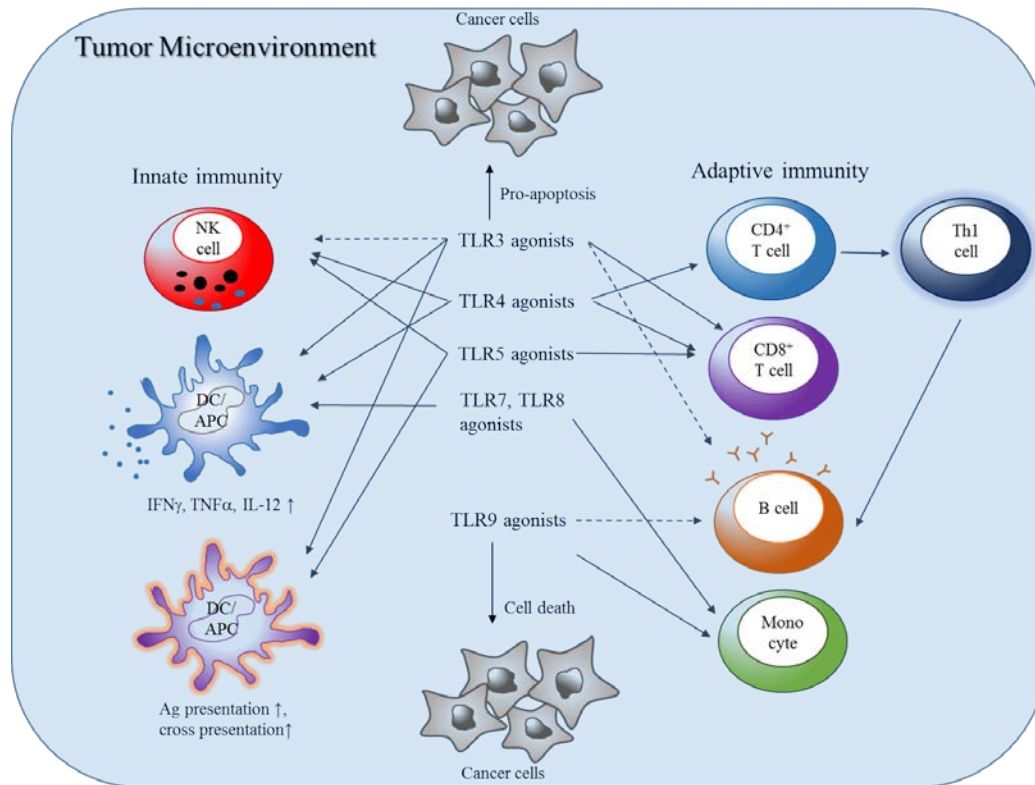
Figure1. Anti-tumor responses by TLR ligands through direct and indirect effects

X.3.1 TLR2

Phylogenetic analysis identified TLR2 along with other TLRs (1, 6 and 10) into the TLR1 family with highly similar primary sequences and cooperate with each other during PAMP recognition [37, 38]. TLR2 can functionally heterodimerize with TLR1, 6 and possibly TLR10 to specifically recognized products from gram positive bacteria, including triacyl lipopeptides and *Mycoplasma fermentans* macrophage-activating lipopeptide (MALP-2). Pam3Cys, a synthetic triacylated lipoproteinis is widely used ligand to activate TLR2 in the laboratory [39].

SMP-105 is a TLR2 agonist developed from the insoluble fraction of the cell wall skeleton (CWS) of *Mycobacterium bovis*. One study has demonstrated that the activation of TLR2 by SMP-105 significantly increased IFNγ producing cells and tumor specific cytotoxic lymphocytes (CTL) in mice inoculated with Lewis lung cancer cells, resulting in a growth suppression of implanted tumors [40]. However, SMP-105 has not yet been trialled, and the use and development of TLR2 agonists as an adjuvant in anti-cancer immunotherapy has not been investigated. This may be at least partly because of regulatory T cell (Treg) activation by TLR2 agonists and

resultant production of the anti-inflammatory IL-10 [41-43]. In contrast, recent studies have shown that TLR2 activation by Pam3Cys can supress Treg activity and promote a Th17 like phenotype shift in multiple sclerosis patients. Overall, this suggests that the outcomes of TLR2 signalling may be contextually and therefore immunobiologically diverse. [44].

CBLB612 is a synthetic lipopeptide that specifically binds to TLR2 and TLR6. However, this molecule was evaluated as only a bone marrow protective agent for cancer patients before and after chemotherapy. A phase II double blind, multi-center study (NCT02778763) was completed on efficacy and safety of this molecule as a monotherapy for neutropenia prophylaxis in breast cancer patients receiving myelosuppressive chemotherapy.

X.3.2 TLR3

TLR3 is an endosomal receptor that recognize double stranded RNA molecules. The primary TLR3 ligand is viral dsRNA. Polyinosinic-polycytidylic acid (polyIC), first developed in 1967, is a synthetic dsRNA molecules used in many studies to activate TLR3 and MDA5 [19, 45]. It has been shown that stimulation with poly-IC induces strong type I interferon production, humoral immunity and Th1 responses. However, the significant toxicity and the degradation by serum nucleases limits its development as a clinical dsRNA adjuvant.

To improve the safety and therapeutic potential, another two poly-IC derivatives, poly-IC12U (Ampligen®) and poly-ICLC (Hiltonol®) were developed and tested in numerous clinical trials as effective adjuvants. The former is a modified poly-IC with shortened half-life and shown to induce lower absolute levels of type I interferon than poly-IC [19]. Early studies showed that Ampligen not only activate NK cell and convert M2 macrophage to M1 counterparts, but also elicit direct cytotoxic effects on cancer cells [19, 23]. However, more interest is currently focused on testing this TLR3 agonist against HIV infection, myalgic encephalomyelitis and chronic fatigue syndrome [46].

Hiltonol® is designed to be highly resistant to serum nucleolytic hydrolysis. Its denaturation temperature can be as a high as 40°C. When administered, the prolonged and enhanced activity of this compound has been shown to induce changes in immune related gene profiles indicative of the activation of multiple canonical innate immune pathways [47]. In early studies, this drug was initially proposed to be used in clinical

trials for treating children with acute leukemia and neuroblastoma [48]. Due to high dose and systemic side effects, more recent clinical trial have transitioned to local administration such as intramuscular (i.m) or subcutaneous (s.c) route. Current studies have demonstrated that inclusion of poly-ICLC with overlapping long peptides (OLP) from a human tumor self-antigen NY-ESO-1 and montanide-ISA-51, was well tolerated and elevated the antigen specific antibody titer and T cell responses in ovarian cancer patients immunized subcutaneously (NCT00616941) [49]. A further study of a sensitive CD154 expression based assay characterised that the major effect of poly-ICLC was achieved through enhancing OLP vaccine-induced CD4$^+$ T cell as an increased IFNγ/IL-4 ratio was detected. However, this study did not assess the influence of using poly-ICLC as a standalone adjuvant [50]. Another very recent study showed that NY-ESO-1 specific IFN producing CD8$^+$ T cells were significantly increased in patients immunised with poly-ICLC than controls without poly-ICLC treatment. [51] (UMIN000007954). Consistent findings were reported in a study of treating low grade glioma (LGG) patients using a subcutaneous emulsified vaccinations of glioma associated antigen (GAA) derived peptide with concurrent intramuscular injections of poly-ICLC (NCT00795457). This project was initially to explore an appropriate strategy for treating immunocompetent patients with slow growth rate gliomas as these patients may exhibit more immunogenicity and gain greater benefit from immunisations than those immunocompromised subjects with high grade gliomas [52]. These results suggest TLR3 agonists as a promising adjuvant to cancer vaccines that target various tumor associated antigens. Nevertheless, large scale trials are required to evaluate the safety and efficacy of TLR3 agonist based cancer therapies.

X.3.3 TLR4

TLR4 was the first TLR identified in mammals and can be activated classically by LPS (also known as endotoxin) from gram-negative bacteria [13]. TLR4 also recognises DAMPS, including heat shock protein 70 [53] and non-histone chromatin binding nuclear constituent HMGB1[54]. These proteins can be released by cancer cells following heat stress or chemo/radiotherapy and act as danger signals promoting anti-tumor immunity by activation TLR4 in DCs. Patients carrying single nucleotide

polymorphisms (SNP) in TLR4 affecting the interaction between TLR4 and HMGB1 relapsed earlier after chemo/radiotherapy than those with the normal TLR4 allele [54].

Monophosphoryl Lipid A (MPL), a derivative of lipid A from gram-negative *Salmonella Minnesota* endotoxin with reduced LPS (TLR4 ligand) toxicity in humans was found to have anti-tumor activity *in vivo* early in the 1960s. Since 1984, this molecule as an adjuvant has been extensively investigated for cancer vaccines in the clinical setting. AS04 (Adjuvant System 04), an aluminium salt and MPL based adjuvant was endorsed by FDA in 2009 as part of Cervarix®. AS04 was also developed by GSK Biologicals in different formulations and used as proprietary adjuvant in numerous trials of cancer vaccine targeting specific TAAs (MAGE-3, MUC-1, Sialyl-Tn and Ras mutant) expressed on multiple cancers types [6, 55].

Bacillus Calmette-Guérin (BCG) is a vaccine primarily developed for the prevention of tuberculosis worldwide [24]. Its anti-cancer potential began to be evaluated in the clinical settings in the 1970-1980s [24]. Testing BCG as monotherapy in human bladder cancer demonstrated either no clinical benefit or were proven inconclusive due to the small cohort sizes. Since, 1977 intravesical instillation of BCG has been the "gold standard" treatment for patients with *in situ* or non-muscle invasive bladder cancer. Several clinical trials listed were designed to compare BCG as a single agent with combination therapy. Successful trials using BCG for melanoma therapy demonstrated better prognosis overall when BCG was combined with a melanoma cell vaccine or in combination with topical treatment of 5% imiquimod, a cream formulated TLR7/8 agonist [56]. The non-specific protection by BCG was shown to be mainly through activation of TLR2 and TLR4 in macrophages and DCs, inducing strong cytokine and chemokine production such as IFNγ, IL-2 and TNFα. However, BCG has been found to activate $CD4^+CD25^+$ Treg cells and promote TGFβ and IL-10 secretion, which could be the reasons of unfavourable results in some trials [56-58].

Picibanil (OK-432), a lyophilized preparation of *Streptococcus. pyogenes* that is approved in Japan for the treatment of cervical cancer, gastric cancer and oral cancer [59]. Studies using this compound in other malignancy appears still active [60-62].

GLA-SE (G100), a synthetic glucopyranosyl lipid A (GLA) which is an oil-in-water emulsion (ES) is a novel TLR4 agonist, and this particular formulation allows to maximise the activation of multiple immune related signalling pathways [63]. A

recent preclinical study showed that intratumoral injection of G100 three times a week significantly suppressed tumor growth and resulted in 60% CR (complete tumor regression) in an A20 lymphoma tumor model via a CD8^{+} T cell dependent manner. Gene profiling analysis further demonstrated upregulation of broad immnune related genes, though also including T-cell exhaustion marker such as CTL4, LAG3 and PD-L1 in G100 treated tumor. These data provide a rationale for co-administration of this TLR4 agonist with checkpoint blockade therapy to gain a potential synergistic effect [64] and support the current on-going clinical trial of G100 for patients with non-hodgkin's lymphoma as a single agent or in combination with pembrolizumab, a humanized antibody against PD-1 (NCT02501473). Furthermore, a single injection of G100 subcutaneously prior to tumor inoculation resulted in reduction of metastatic development of a mammary adenocarcinoma and a colon cancer cell model in both rats and mice with no adverse effects. The anti-tumor effect of G100 appeared to be mainly associated with enhanced activation of NK cells [65]. A pilot clinical trial of intratumoral injection of G100 as monotherapy for patients with resectable Merkel cell carcinoma in a neoadjuvant setting exhibited an acceptable tolerability and increased CD8^{+} T cell antitumor activity [59, 66]. Additionally, in order to trigger a potent tumor antigen-specific anti-tumor response, G100 was also formulated with recombinant NY-ESO-1 protein, and developed to be another immunogenic agent named ID-G305. A novel "priming-boost" combination approach called CMB305 is to sequentially dose LV305 (an *invivo* DC targeting vector expressing the NY-ESO-1 gene) and ID-G305. This recipe allows to synergistically induce multiple level of anti-tumor immune effects. Of note, CMB305 does not require patient-specific manufacturing or *ex vivo* manipulation of patient samples. The Phase Ib open label, multi-centre trial designed to evaluate the safety, tolerability, immunogenicity, and preliminary clinical efficacy of CMB305 in patients with NY-ESO-1 positive tumors is currently recruiting (NCT02387125).

X.3.4 TLR5

Flagellin protein, a constituent protein of bacterial flagella is the only known natural ligand to activate TLR5. Formulation using liposomal engrafted synthetic peptide containing flagellin fragments can induce DC maturation *in vitro* and *in vivo* [25]. In a study employing a mouse xenograft melanoma model, a vaccine formulated with both N- (9Flg and 42Flg) and C- (10Flg and 11Flg) terminal flagellin peptide engrafted

Ovalbumin (OVA) liposomes suppressed lung metastasis in mice inoculated with B16-OVA cells compared to control mice [67]. More interestingly, TLR5 agonists lack induction of self-amplified driving cytokines, such as TNF-α, IL-1β and IL-2, which make it fingerprint as a safe adjuvant for systemic administration [26].

Entolimod (CBLB502), a pharmacological optimised flagellin derivative, has revealed antitumor effects in numerous mouse tumor models [26, 68-71]. Craig et al. also showed that activation of TLR5 signaling by systemic administration of Entolimod as a single agent inhibited at least two types of murine tumor metastases to high TLR5 expression organs such as lung and liver. These antitumor effects were initiated through a CXCR3 dependent NK-DC-CD8$^+$ T cell axis [26]. One phase I clinical trial has been completed to determine the safety and preliminary evidence of efficacy using Entolimod (i.m or s.c) in patients with late stage solid tumors (NCT01527136). Overall, the treatment with Entolimod was well tolerated with only common adverse events such as fever, transient hypotension and hyperglycemia.

Interestingly, Entolimod has been shown a protective effect against renal dysfunction in a murine model of acute renal ischemic failure as well as in an ulcerative colitis model [72, 73]. Furthermore, this compound showed radioprotective activity in mouse and primate models without reducing tumor radiosensitivity [74]. Further preclinical study identified liver and gastrointestinal tract as the major target organs of this molecule [75]. These evidence supported current clinical studies and raise considerable interest to test Entolimod in development as a radiation countermeasure in emergency severe condition such as acute radiation syndrome or radiation sickness from radiotherapy against cancer. These findings allow Entolimod to be a versatile player in cancer therapy.

Another TLR5 agonist, M-VM3 (Mobilan), is currently in two clinical trial (NCT02654938, NCT02844699) for prostate cancer. Mobilan was designed as a recombinant non-replicating adenovirus encoding human TLR5 and its specific agonistic ligand, flagellin. Delivery of this system into tumor cells would render an autocrine activation of TLR5 and result in subsequent strong adaptive anti-tumor immune responses. Certain human tumors expressing the Coxsackievirus and adenovirus receptor (CAR) such as prostate cancer and several tumors of female reproductive system will be the primary targets using this TLR5 agonist based system [76].

X.3.5 TLR7/8

TLR7 and 8, both expressed in the endosomes/lysosome, are receptors for ssRNA, especially U or GU-rich oligoibonucleotides [77]. They share high sequence homology and predominately overlap in the ligands they can each respond to. In humans, these TLRs are abundantly expressed in multiple subsets of human DCs. Stimulation of TLR7 and 8 with their agonists significantly augments multiple subset DC maturation, Th1 cellular immunity, cross-presentation and humoral immunity [78, 79]. Of note, conjugation of TLR7/8 agonist rather simply mixing with antigens has been demonstrated more effective to generate CD8$^+$ immunity [17, 80].

Resiquimod, a TLR7/8 bispecific agonist is a prototypical imidazoquinoline molecule [17]. Early studies showed that soluble molecules like resiquimod distribute quickly from the site of injection throughout the body and fail to induce local immune activation, thereby limiting its clinical utility. To resolve this problem, prototypical imidazoquinolines were formulated as a dermal cream [17]. Aldara®, imiquimod 5% Cream is the one of the only three FDA approved commercialised small molecule TLR agonist for HPV-mediated external genital warts, superficial basal cell carcinoma and actinic keratosis [24]. In recent clinical trials, Imiquiod cream has been further exploited as standalone or in combination with various anti-tumor therapies including chemo/radio- or laser therapy in various human cancers [17]. A study of topical Imiquimod in breast cancer patients with skin metastasis reported that 2 of 10 patients achieved a partial response with histologic evidence of immune mediated tumor regression. The treatment was well tolerated with exception of frequently local adverse events [81]. In contrast, another randomized controlled trial (NCT00066872) conducted at 12 centres in the UK on 501 participants reported that patients with nodular and superficial basal cell carcinoma treated with Imiquimod 5% cream was superior to excision surgery [82]. Considering the diversity of subtype of this skin cancer, to determine surgical or non-surgical modalities alone or combination needs to be optimized by future investigation [83, 84]. Futhermore, in a completed two-part randomised trial on high risk melanoma patients, NY-ESO-1 antigen emulsified in Montanide was intradermally injected, followed by topical application of 0.2% Resiquimod gel or placebo to the vaccine site. Although this formulation with topical Resiquimod was safe, the clinical outcome reveals no significant differences between study groups as addition of topical Resiquimod was not sufficient to induce consistent specific CD8$^+$ T cells. The reason could be that topical application of the TLR agonist

may fail to absorb adequately and activate diverse DC populations in deeper skin layers [85], highlighting the selection of administration route for optimal efficacy using TLR agonists.

3M-052, a novel lipid modified imidazoquinoline was developed and evaluated as part of a conventional vaccine formulation. Compared to Resiquimod, 3M-052 induced a prolonged response locally with diminished systemic inflammation. It was also evaluated as an adjuvant in many vaccine models such as alum suspensions, liposome formulation and oil-in-water emulsion [17, 86, 87]. In a preclinical study, Singh et al. showed that intratumoral injection 3M-052 in mouse melanoma and prostate tumor models suppressed local and distant tumor growth via not only promoting tumor specific CD8$^+$ T cells but also shifting M2 macrophages to M1 phenotype. 3M-052 has also exhibited synergic effects with checkpoint inhibitor therapy using CTLA4 and PDL-1 antibodies [88].

X.3.6 TLR9

TLR9 is expressed in the endosome of specific immune cell types - plasmacytoid DC and B cells where they recognize bacterial or viral DNA containing unmethylated cytosine-guanine (CG) dinucleotides motifs[89]. Activation of TLR9 through the signaling of MyD88 leads to activation of interferon regulatory factor (IRF)7, resulting in expression of Type I IFNs [90].

CpG-7909 (PF-3512676, Promune®), a Class B CpG, is the most extensively studied single-stranded CpG ODN. Unfortunately, two phase III clinical studies for advanced non-small cell lung cancer reported that addition of CpG-7909 to chemotherapy gained no improvement to either overall or progression-free survival [91, 92]. Although phase I/II studies examining CpG-7909 in combination therapies were completed in in various human cancer such as B-cell lymphoma (NCT00185965), metastatic breast cancer (NCT00824733) and esophageal cancer (NCT00669292) no trials further assessing this molecule for cancer therapy are active.

MGN1703, a covalently closed natural DNA molecule is a novel TLR9 agonist, which belongs to a different family called dSLIM (Double Stem Loop Immunomodulatory) [89, 93]. While CpG-7909 mainly stimulates B cells and may cause several CG-motif independent immune responses like IL-8 induction, dSLIM elicits significant IFN-α induction and broad activation of human immune cells *in*

vitro [89]. In a phase II study of 59 patients with metastatic colorectal cancer treated with the first-line chemotherapy bevacizumab (anti-VEGF-A), patients who also received MGN1703 showed a superior progression free survival (PFS) by (NCT01208194) [94-96]. A pivotal phase III trial has been designed to further investigate these data and are currently recruiting patients (NCT02077868). This compound has also been tested in a phase I trial to determine the highest tolerable dose in combination with ipilimumab (anti-CTLA-4) to patients with advanced solid tumours (NCT02668770) and another study in patients with small cell lung cancer (SCLC) (NCT02200081).

SD-101, another synthetic CpG molecule was tested by intratumoral co-administration with ipilimumab after local radiation therapy in patients with low-grade, recurrent B-cell lymphoma (NCT02254772). The primary objective was to identify the best dose of intratumoral ipilimumab with TLR9 agonist in patients to augment different phases of anti-tumor immune responses as shown in previous preclinical studies [97, 98]. However, the result has not been released yet. Another trial using this molecule combined with ibrutinb (also known as Imbruvica, a targeted inhibitor for Bruton's tyroinse kinase) and intratumoral radiotherapy for low-grade follicular lymphoma is currently listed as recruiting (NCT02927964) in order to re-evaluate adverse event during this heavy combination therapy.

X.4 Conclusion remarks

Coupled with chimeric antigen receptor therapy [99] and immune checkpoint blockade [100], engagement of manipulating TLR signaling has drawn considerable interest as a treatment modality in the cancer immunotherapy field. Particularly, synthetic TLRs agonists have been actively exploited for their safety and clinical efficacy in various therapeutic settings. Substantial evidence demonstrates that TLR agonists are potent immunostimulators and enhance natural or therapy-triggered anti-tumor immune responses. Despite the wealth of research in the cancer immunotherapy field, the past few years has witnessed a steady decrease in the number of clinical trials using TLR agonists as cancer treatments, be that unimodal or as adjuvants to cancer vaccines [59]. There may be some reasons for this. Firstly, most TLR ligands initiate complex signaling cascade and may influence various cell types in cell dependent manners, of which we haven't fully elucidated. Immune cells, cancer cells and tumor stromal cell differ in their specific TLRs expression and may all contribute

to biological consequences of TLR activation [101]. Non-inflammatory roles of TLRs in tumor progression such as regulation of apoptosis and proliferation in context of chronic inflammation and carcinogenic condition has been addressed [9]. Secondly, recent studies reveal that TLR activation can exert not only immunostimulatory effects but also immunosuppressive effects by regulating IL-10, Treg activity and PD-L1 expression [102-104]. In addition, as with many other anti-tumor agents in general, underperformance of TLR agonist used in some trials may relate to the fact that patients recruited in most studies are in late-stage disease. Metastatic tumours, are aberrant in multiple signalling pathways and have mutations in various key genes regulating cellular functions may all contribute to drug resistance. Additionally immune system depression as a consequence of late stage cancer may impede any effective induction of anti-tumor responses. Last but not least, the delivery system, type of tumor associated antigen, type of TLR agonist and/or other adjuvants, schedule, route and site of administration need to be carefully considered and further investigated to acquire optimal activation and specificity [19]. Recent convergence of large scale sequencing, cancer biology and bioinformatics allows researchers to tailor the strategy of priming the immune system against multiple patient-specific neoantigens (tumor mutation-derived antigens) [105, 106]. Additionally, there is scope for these formulations of specific TLR agonists to be personalised for specific patients' tumours. Combination therapies based on stratification of patients by their immune state may lead to better targeted trials using TLR agonists.

Overall, basic research and clinical trials provide a strong rationale for using TLR agonists as adjuvants to cancer treatments.

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