Vaccine adjuvants as potential cancer immunotherapeutics

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Received 18 February 2016, *accepted* 14 March 2016

Abstract

Accumulated evidence obtained from various clinical trials and animal studies suggested that cancer vaccines need better adjuvants than those that are currently licensed, which include the most commonly used alum and incomplete Freund's adjuvant, because of either a lack of potent anti-tumor immunity or the induction of undesired immunity. Several clinical trials using immunostimulatory adjuvants, particularly agonistic as well as non-agonistic ligands for TLRs, C-type lectin receptors, retinoic acid-inducible gene I-like receptors and stimulator of interferon genes, have revealed their therapeutic potential not only as vaccine adjuvants but also as anti-tumor agents. Recently, combinations of such immunostimulatory or immunomodulatory adjuvants have shown superior efficacy over their singular use, suggesting that seeking optimal combinations of the currently available or well-characterized adjuvants may provide a better chance for the development of novel adjuvants for cancer immunotherapy.

Keywords: β-glucan, combination, CpG ODN, STING, TLR

Introduction

Despite intense cancer research and the culminated advances over the last few decades, cancer still remains one of the major health problems, impacting millions of people worldwide and suggesting the need for novel approaches to cancer treatment. Among the emerging therapeutic strategies, cancer immunotherapy, where one's own immune system is exploited against the tumors, is one of the outstanding treatment approaches. The main purpose of cancer immunotherapy is overcoming the immunosuppression in the tumor microenvironment so that the immune cells can effectively eliminate the tumors without causing intolerable side-effects (1). To achieve this goal, cancer immunotherapy may utilize various means including cytokines (e.g. GM-CSF and IL-2), oncolytic viruses, checkpoint inhibitors [e.g. cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed death 1 (PD-1) and/or indoleamine 2,3-dioxygenase (IDO)], adoptive cell transfers (e.g. adoptive T-cell therapy) and therapeutic cancer vaccines (DNA- or RNA-based vaccines or adjuvanted peptide or protein vaccines), the lattermost being the focus of this review (2–4).

In the 19th century, the first successful attempts of cancer immunotherapy were made by Dr William Coley who thought to inject inoperable tumors with inactivated bacteria (*Streptococcus pyogenes* and *Serratia marcescen*)—the so-called Coley's toxin (5). Later, it was found that the anti-tumor effect of Coley's toxin was due to the stimulation of multiple pattern-recognition receptors (PRRs) including TLRs, such as TLR4 (6). Coley's study was followed by a similar attempt to use BCG, a live attenuated *Mycobacterium bovis* strain, for treating bladder cancer. In fact, intravesical BCG injection is still a US Food and Drug Administration (FDA)-approved immunotherapy for treating superficial bladder cancer (7, 8).

Since then, characterization of various tumor-specific antigens together with the approval of some cancer vaccines by the FDA strongly encouraged the field of cancer immunotherapy, making cancer immunotherapy, especially cancer vaccines, a promising alternative or a crucial part of the combinatorial treatment approaches including radiotherapy, chemotherapy or surgery (1).

For example, Provenge is the first FDA-approved therapeutic prostate cancer vaccine developed by harvesting and modifying the dendritic cells (DCs) of the patient's own peripheral blood so that they could attack and eliminate the prostate cancer cells (9). On the other hand, Gardasil and Cervarix are considered prophylactic cancer vaccines, as they have been developed for preventing human papilloma virus (HPV) infection that is associated with almost 70% of cervical cancer

cases ([10], [11]). Yet Rosenberg *et al.* reported that in a clinical trial with high numbers (440) of cancer patients, the efficacy of the cancer peptide vaccines was extremely low (2.6%) based on the selection of nonimmunogenic antigens or lack of powerful adjuvants capable of overcoming the immuno-suppression in the cancer patients ([12]).

Therefore, a potent adjuvant is a crucial component of cancer vaccines, as it can break the immunotolerance in the tumor microenvironment to aid in the elicitation of potent anti-tumor immune responses. In this review, we introduce novel adjuvants used either as cancer vaccine adjuvants or as immunotherapeutic agents in pre-clinical and clinical trials. Also, we discuss the limitations and advantages of these adjuvants by giving insights into the types of immune responses that are elicited by each of the adjuvants.

Types of adjuvants

The term adjuvant comes from the Latin word 'adjuvare', meaning help. Therefore, adjuvants help vaccines improve the antigen-specific immune response by serving as, or inducing, damage-associated molecular patterns (DAMPs) and/or pathogen-associated molecular patterns (PAMPs) that activate various PRRs of innate immune cells including TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs), retinoic acid-inducible gene I (RIG-1)-like receptors (RLRs) or C-type lectin receptors (CLRs) ([6], [13]). Sensing of the DAMPs or PAMPs, such as microbial components (e.g. microbial DNA or LPS, by innate immune cells initiates a cascade of immune responses resulting in the elicitation of potent innate and adaptive immune responses against the invading pathogens or tumor cells ([6], [13]). Alternatively, adjuvants may function as delivery systems that can efficiently deliver the antigen into the antigen-presenting cells (APCs) to promote the elicitation of antigen-specific immune responses ([14], [15]). In Table 1, we provide an overview of adjuvants with their classification and mechanisms of action.

Conventional adjuvants serving as delivery systems

Virosomes

Virosomes are spherical viral particles without viral genetic material and the nucleocapsid, therefore incapable of replicating and infecting. Yet, as virosomes retain the ability of the parental virus strain to fuse, they can be engineered to carry tumor-specific antigens and/or adjuvants into the APCs to improve the efficacy of cancer vaccines ([17]). Influenza virosomes are one of the widely studied delivery tools used in a number of cancer vaccines and have been shown to induce tumor-specific antibody, T cell and especially CTL responses ([1], [18]). For instance, results from a phase I clinical trial, involving Her/neu[+] metastatic breast cancer patients, demonstrated that therapeutic cancer vaccine formulated with the Her/neu peptide-containing influenza virosomes is well tolerated and capable of inducing Her/neu-specific antibody and cellular immune responses in addition to decreasing the number of T_{reg} cells in the peripheral blood of the vaccinated patients ([19]).

Liposomes

Liposomes are synthetic phospholipid vesicles that can be used for antigen or adjuvant delivery systems. Although their versatility, biocompatibility and biodegradability make liposomes potential candidate delivery systems for use in vaccines, use of the liposomes for human applications is restricted due to concerns about stable manufacturing of vaccine-grade liposomes as well as high cost ([14]).

However, several animal studies using liposomes as adjuvant or tumor-specific antigen delivery agents showed that liposomal cancer vaccines have superior anti-tumor efficacy over non-liposomal vaccines ([14], [20], [21]). For example, in the mouse model of neuroblastoma, liposomal delivery of cytosine guanine dinucleotide oligodeoxynucleotides (CpG ODNs) specifically to the tumors has been shown to provide potent anti-tumor effects, whereas the CpG-alone group failed to induce such an anti-tumor effect ([22]). Moreover, liposomal vaccines containing basic fibroblast growth factor and the adjuvant, monophosphoryl lipid A (MPLA), have been shown to induce anti-tumor immunity by inducing tumor-specific antibody and T_h1-type immune responses in mice challenged with Lewis lung carcinoma cells ([20]). In addition, liposomal delivery of the lipid antigen, α-galactosylceramide, provided potent anti-tumor immune responses to prevent lung metastasis in the 65% of B16 F10-tumor-bearing mice by specifically activating the NKT cells in the spleen ([21]).

Clinical trials in follicular lymphoma patients using liposomes as a vaccine delivery agent for the tumor-specific antigen (Id) demonstrated that liposomal vaccines are potentially safe and capable of inducing long-lasting antigen-specific CD4^{+} and CD8^{+} T-cell responses ([23]).

Table 1. Classification of adjuvants on the basis of their modes of action

Class	Adjuvant	Immune response
Delivery systems	Liposomes, virosomes	B, T_h1, T_h2, CTL
	Emulsions: MF59, Montanide	B, T_h1, T_h2, CTL
	Saponin based: ISCOMs, QS-21	B, T_h1, CTL
	Mineral salts: alum, AS04	B, T_h1, T_h2
Immunostimulants	TLR ligands: Poly I:C, MPL, GLA, imiquimod, CpG ODN	B, T_h1, T_h2, NK, CTL
	Saponin based: ISCOMs, QS-21	B, T_h1, CTL
	Polysaccharides: chitin, chitosan ([16]), β-glucan	B, T_h1, T_h2, NK, CTL
Combinations	TLR9 + STING ligands: K3 CpG + cGAMP	B, T_h1, NK, CTL
	Adjuvant Systems: AS02, AS04, AS15	B, T_h1, T_h2

Immune stimulating complexes

Immune stimulating complexes (ISCOMs) are open cage-like particles with a diameter of ~40 nm that are composed of Quillaia saponins, cholesterol and phospholipid. Because of their structure, they can achieve efficient antigen delivery into cells, resulting in the induction of both cellular and long-lasting humoral immune responses in an antigen-specific manner (24). The mechanisms of action of ISCOMs involve direct interaction with DCs to promote antigen cross-presentation, thereby allowing the induction of robust antigen-specific CD4+ and CD8+ T-cell responses (25). In addition, recent reports indicated that the ISCOMATRIX adjuvant can activate the NLR-family pyrin-containing protein 3 (NLRP3) inflammasome pathway to cause IL-1β and IL-18 production in mice. However, IL-18, but not IL-1β, is involved in the mechanisms of action of ISCOMATRIX as a vaccine adjuvant. Also, TNF-α is partially involved in adjuvant activity of ISCOMATRIX by priming the NLRP3 inflammasome for subsequent IL-18 production (26).

ISCOMs have been used in clinical trials involving patients with NY-ESO-1+ tumors as a cancer vaccine adjuvant. These studies showed that the vaccine, containing ISCOMATRIX adjuvant and the recombinant NY-ESO-1 protein, is safe and capable of inducing potent cellular and humoral immune responses that can provide superior clinical benefits to the vaccinated patients (27, 28). In addition, pre-clinical trials in mice bearing ovalbumin-expressing Panc02 (also known as Pan02) tumors revealed that the use of ISCOMs together with the TLR9 ligand, CpG ODN, as adjuvants provided superior anti-tumor immunity and protection to the tumor-bearing mice after re-challenge with the tumor, suggesting that the development of more potent and promising cancer vaccine adjuvants can be achieved by the combination of ISCOMs with the currently available adjuvants (29).

QS-21

QS-21 is another saponin-based adjuvant consisting of the saponins extracted from the Chilean Soap Bark Tree (*Quillaja Saponaria*). QS-21 is able to induce antigen-specific antibody, CTLs and T_h1-type immune responses, as well as having low toxicity (30). Recently, QS-21 was shown to activate the NLRP3 inflammasome to induce IL-1β and IL-18 production in mice. Indeed, NLRP3-deficient mice showed increased antigen-specific antibody and T-cell responses compared with wild-type mice, suggesting a negative regulatory role for the NLRP3 inflammasome in QS-21-induced immune responses (31). Clinical trials involving breast cancer or prostate cancer patients showed that QS-21 is a well-tolerated and immunogenic adjuvant capable of inducing antigen-specific antibody responses (30, 32, 33). Furthermore, the combined use of QS-21 with different adjuvants, such as MPLA and CpG ODN, have been reported to improve its immunogenicity, and thus current efforts are being made to develop the optimal combinations of QS-21 with different adjuvants to be used in cancer vaccines (30, 32).

Mineral salt (alum)

Among the limited number of adjuvants that have been approved for human use, the most commonly used adjuvants are aluminum based and include aluminum phosphate or hydroxide. So far, alum has been used as an adjuvant for a variety of vaccines including the ones against diphtheria plus pertussis plus tetanus, against HPV, against *Haemophilus influenzae* type B and against hepatitis A virus (34). Despite the controversial results about the mode of action of alum adjuvants, alum is thought to function by forming antigen depots at the injection site, from where antigen is gradually released to allow prolonged interaction of the antigen with the immune cells and therefore causing the induction of sustained antigen-specific B- and T-cell responses for a long time (35, 36).

Moreover, our previous studies suggested an additional role for alum-induced cell death in the mechanisms mediating the adjuvant activity of alum. Particularly, our studies showed that host DNA, which is released because of the alum-induced cell death, is sensed by the immune system to induce IgE-type antibody responses in an interferon regulatory factor 3 (IRF3)-dependent manner, whereas IgG1-type antibody responses are induced in an IRF3-independent manner. Hence, alum-induced host DNA release caused by cell death can function as a DAMP and mediate the adjuvant activity of alum (37).

Several reports showed that alum adjuvants can induce sustained antigen-specific B-cell responses (34, 35), and when used in combination adjuvants, such as AS04, a mixed antigen-specific T_h1/T_h2 response was reported to be induced in human cells and mice (38). Yet, alum adjuvants fail to induce strong T_h1-type and cellular immune responses that have been shown to enhance anti-tumor immune responses (34, 39). Therefore, the use of alum adjuvants in therapeutic cancer vaccines is limited (34). However, recent clinical trials in advanced non-small cell lung cancer (NSCLC) patients revealed that when used in a therapeutic cancer vaccine targeting the tumor-associated ganglioside, NeuGcGM3, alum is capable of inducing high IgM and IgG-type antibody responses against NeuGcGM3, which was correlated with the prolonged survival of vaccinated patients (40).

Water-in-oil emulsions (Montanide)

Montanide-based adjuvants belong to the group of water-in-oil emulsion adjuvants that are prepared by using the surfactants from the mannide monooleate family (41). Similar to alum, Montanide adjuvants have been shown to act via depot formation at the site of injection to allow gradual antigen release and the induction of prolonged immune responses (42).

Depot formation by the Montanide ISA 51 [also known as incomplete Freund's adjuvant (IFA)] was reported to impair tumor-specific CTL responses via the induction of IFN-γ-mediated and Fas ligand-dependent apoptosis, which was driven by the persistent antigen at vaccination sites (43). Nevertheless, Montanides include emulsion adjuvants, ISA 720 and ISA 51 (IFA), which have been used in the vaccines for clinical trials (41, 44), and ISA 206 and ISA 50 V that have been used in animal vaccines only (45). Montanides ISA 720 and ISA 51 have been used as cancer vaccine adjuvants in clinical trials involving several different types of cancer, such as melanoma and NSCLC, and they have been reported to induce antigen-specific antibody and T-cell responses correlated with prolonged survival of the

immunized patients, suggesting that the Montanide-based adjuvants might be promising adjuvants for cancer vaccines (46, 47).

Oil-in-water emulsions (MF59)

MF59 is a squalene-based oil-in-water emulsion adjuvant prepared by emulsifying the squalene using the surfactants, Tween 80 and Span 85, to form stable and uniform nanodroplets with 250 nm diameter (42, 48). The mechanisms of action of MF59 include induction of ATP release from the muscles at the vaccination site, which can further enhance antigen-specific immune responses by acting as a DAMP (49). MF59 was first licensed as an influenza vaccine (Fluad) adjuvant in Europe in 1997, and it has been shown to have a good safety profile and immunogenicity (50).

MF59-adjuvanted vaccines are able to provide protective immune responses against influenza in both the elderly and children by inducing antigen-specific antibody and a mixed T_h1/T_h2-type immune responses (48, 50). Yet the use of MF59 is limited for cancer vaccines, as it can induce not only the advantageous T_h1-type responses but also the undesired T_h2-type immune responses for anti-tumor immunity. However, the combination of MF59 with CpG ODN has been reported to induce melanoma-specific anti-tumor immune responses and prolong the survival of tumor-bearing mice in a mouse melanoma model when used as an adjuvant in the therapeutic cancer vaccine (51).

Innate immune stimulators

CLR ligands

CLRs, such as Mincle, Dectin-1, Dectin-2 and CLEC9A, contain carbohydrate-binding domains, through which they recognize various carbohydrate motifs on microbes. For instance, β-glucan, one of the well-studied Dectin-1 ligands, is abundantly found in the cell walls of bacteria, yeast and fungi. Recognition of β-glucan by Dectin-1 activates the spleen tyrosine kinase (Syk)–NF-κB axis to induce pro-inflammatory cytokine production (52). Pre-clinical studies in mice revealed that β-glucan can improve anti-tumor immune responses by activating the complement system and recruiting tumoricidal granulocytes in addition to the activation of the Dectin-1 signaling pathway (53, 54). On the other hand, although some clinical trials, conducted in Japan and China, demonstrated the anti-tumor effect of β-glucan in carcinoma and leukemia patients, there are no high-quality data obtained from clinical trials utilizing β-glucan as an anticancer agent or vaccine adjuvant (54, 55).

RLR ligands

RLRs, RIG-I and melanoma differentiation-associated gene 5 (MDA5), are cytoplasmic RNA sensors containing caspase activation and recruitment domains (CARDs), through which they can activate the interferon promoter stimulator 1 (IPS1)–inhibition of IκB kinase/tumor necrosis factor receptor-associated factor family member-associated NF-kappa-B activator (TANK)-binding kinase 1 (TBK1) axis (i.e. signaling via IPS1 and IκB kinase or TBK1) to induce type I interferons (52). In addition, liposomal delivery of the synthetic double-stranded RNA (dsRNA), polyribosinic:polyribocytidic acid (Poly I:C) into melanoma cells, has been reported to cause IPS1- and type 1 IFN-dependent apoptosis both *in vitro* and *in vivo* (the explanted mouse melanoma model) (56). Yet, as Poly I:C is recognized not only by RLRs but also by TLR3, the anti-tumor effect of Poly I:C will be further discussed in the 'TLR3 ligands' section.

STING ligands

Stimulator of interferon gene (STING) is an endoplasmic reticulum-resident adaptor molecule capable of activating the TBK1–IRF3 axis to induce type I interferon production in response to intracellular DNA. Indeed, the adjuvant activity of DNA-based vaccines was shown to be mediated by STING stimulation, which can elicit antigen-specific CTL responses (57, 58). In addition to acting as potent vaccine adjuvants, several STING ligands have been shown to exert anti-tumor effects. For example, the synthetic STING ligand 5,6-dimethylxanthenone-4-acetic acid (DMXAA) achieved robust anti-tumor immune responses in mice but not in humans because of the selective binding of DMXAA to mouse STING (59, 60). In addition, cyclic dinucleotides, such as cyclic guanosine monophosphate (cGAMP), cyclic diguanylate and cyclic diadenylate, have been shown to act as STING agonists capable of boosting anti-tumor immune responses when used as a vaccine adjuvant or an immunotherapeutic agent (61–63). However, no clinical trials have been conducted to evaluate the immunotherapeutic potential of cyclic dinucleotides in cancer patients yet.

TLR ligands

TLRs recognize PAMPs that are conserved among a variety of pathogens as well as recognizing the DAMPs released by dying or stressed cells. This recognition initiates an inflammatory response that will aid in elimination of the cancerous or infected cells within the host (6). The mechanisms by which TLR signaling can break the immunotolerance to tumor-associated antigens in APCs involve up-regulation of co-stimulatory molecules, such as CD80, CD86 and CD40, and pro-inflammatory cytokine production, leading to the generation of potent innate and the subsequent adaptive immune responses against tumors (64, 65). Particularly, among the TLR-induced pro-inflammatory cytokines, type I interferons have been reported to improve anti-tumor immunity (66, 67). Thus, several reports showed that TLR ligands have the potential to be used both as cancer vaccine adjuvants and as immunotherapeutic agents (67). Table 2 summarizes the applications and current status of TLR ligands in cancer immunotherapy.

TLR1–TLR2 ligands. Unlike most TLRs that function alone, TLR2 functions by dimerizing with TLR1 or TLR6. Whereas the TLR1–TLR2 heterodimer is responsible for sensing triacylated lipopeptides of mycoplasma or Gram-negative bacteria origin, TLR2–TLR6 heterodimers sense diacylated lipopeptides of mycoplasma and Gram-positive bacteria origin (6). TLR2 ligands have been reported to function both as potent cancer vaccine adjuvants and as immunotherapeutic agents in several different mouse cancer models (69, 80, 81). For instance, Zhang *et al.* showed that systemic treatment of tumor-bearing mice with a synthetic TLR1–TLR2 agonist (bacterial lipoprotein) can suppress tumor growth by suppressing T_{reg} cell

Table 2: Applications and current status of TLR ligands in cancer immunotherapy

TLR	Ligands	Clinical status and type of immunotherapy	Outcomes	References/clinical trial identifiers
TLR1–2	Bacterial lipoprotein (BLP)	Pre-clinical (systemic adjuvant injection)	Limitation of suppressive T_{reg} function to enhance tumor-specific CTL responses	(68)
	Pam_3CSK_4	Pre-clinical (tumor cell treatment with adjuvant before inoculation)	Enhancement of immunogenicity of B-cell lymphoma cells	(69)
TLR3	Poly I:C	Pre-clinical (systemic adjuvant treatment after peptide vaccination)	Induction of tumor-specific CTL and NK cell responses	(70)
	Poly ICLC (Hiltonol)	Case study (repetitive intratumoral and intramuscular adjuvant injection)	Significant tumor regression and prolonged survival in a patient with advanced facial embryonal rhabdomyosarcoma	(71)
		Phase I–II (intramuscular cancer vaccine adjuvanted with Poly ICLC)	Expected: improvement of the efficacy of peptide vaccine in glioblastoma patients	NCT01920191
TLR4	MPL in liposomal emulsion	Pilot study (vaccine adjuvant)	Induction of tumor-specific humoral and cellular immune responses in colorectal cancer patients	(72)
	GLA-SE (glucopyranosyl lipid A-stable emulsion)	Phase I (intratumoral adjuvant injection)	Evaluation of safety and efficacy in metastatic sarcoma patients	NCT02180698
	AS04 (alum + MPL)	Licensed for prophylactic HPV vaccines	Protection from HPV-related genital and vulval precancerous lesions and genital warts	(10)
TLR7	Imiquimod	Approved for treatment of actinic keratosis and basal cell carcinoma	Tumor regression via DC, CTL and NK cell recruitment upon topical application	(73)
	TMX-101	Phase II (intravesical adjuvant injection)	Evaluation of safety and efficacy in bladder cancer patients	NCT01731652 (74)
TLR9	PF-3512676 (CpG ODN)	Phase II (subcutaneous adjuvant injection)	Induction of type I interferons and NK cell-mediated anti-tumor immunity in melanoma patients	(75)
	MGN-1703/dSLIM	Phase II (subcutaneous adjuvant injection)	Evaluation of safety and efficacy in small-cell lung cancer patients	NCT02200081
	K3 CpG+cGAMP	Pre-clinical (intratumoral injection)	Anti-tumor immunity induction via CTL and NK cells in EG-7 and B16 F10 models, respectively	(76)
	K3-SPG (K3 CpG wrapped with non-agonistic Dectin-1 ligand)	Pre-clinical (vaccine adjuvant)	Induction of antigen-specific humoral and cellular immunity, especially CTL	(77)
	AS02 (MPL+QS-21 in an oil-in-water emulsion)	Phase I/II (vaccine adjuvant)	Induction of MAGE-3-specific B- and T-cell responses in patients with MAGE-3-expressing tumors (mostly melanoma)	(78)
	AS15 (MPL+QS-21+ liposome + CpG ODN)	Pilot trial (vaccine adjuvant)	Induction of MAGE-A3-specific CD4+ T-cell responses in MAGE-A3-positive melanoma patients	(79)

function and enhancing the CTL activity in explanted tumor models of melanoma, carcinoma and leukemia (68).

TLR3 ligands. TLR3 is an endosomal TLR that senses RNA virus infection in the cells by recognizing intracellular dsRNA (6). Poly I:C is a synthetic TLR3 ligand that has been reported to function as a potent type 1 adjuvant capable of activating antigen-specific antibody, CTL and T_h1 type immune responses (70, 82). However, in addition to activating the TLR3-mediated signaling pathway, as mentioned above Poly I:C was found to activate intracellular RNA sensors, RIG-I and MDA5, which is associated with its toxic effects, such as systemic cytokine storm induction (83, 84).

To overcome these toxic effects of Poly I:C, Matsumoto *et al.* recently developed a modified synthetic dsRNA that binds to TLR3 but not to RIG-I or MDA5. And they showed

that this specific TLR3 ligand can induce potent NK cell- and CTL-mediated anti-tumor immune responses in mouse tumor models (85). Furthermore, polyinosinic–polycytidylic acid stabilized with polylysine and carboxymethyl cellulose (Hiltonol) is another modified Poly I:C stabilized with poly-L-lysine and carboxymethylcellulose for protecting it from degradation by nucleases. Pre-clinical trials in mice showed that when used as an adjuvant, Poly I:C and Poly ICLC can induce potent tumor-specific CTL, NK and NKT cell responses providing significant tumor regression and prolonged survival of tumor-bearing mice (86, 87). On the other hand, clinical trials involving limited numbers of patients with different types of tumors showed that repetitive intramuscular injections of Poly ICLC without any antigen has low toxicity and promotes anti-tumor immune responses (71, 88).

Moreover, another modified nontoxic, good manufacturing practices (GMP)-grade Poly I:C analogue is Poly (I:C$_{12}$C) (Ampligen) developed by introducing regular mismatching bases (G and U) into Poly I:C to make it more susceptible to hydrolysis and concomitantly decreasing its toxicity. Systemic administration (intravenous) of Poly (I:C$_{12}$C) was already shown to be nontoxic and well tolerated in HIV$^+$ patients (89, 90). In addition, clinical trials in patients with metastatic malignancies, such as renal cancer, revealed that Poly (I:C$_{12}$C) can boost anti-tumor immune responses to provide clinical benefit and prolonged survival to patients via mechanisms activating potent NK and T-cell responses (91). Yet large-scale clinical trials are needed to confirm the potency of TLR3 ligands as anti-tumor agents.

TLR4 ligands. TLR4 recognizes highly toxic LPS structures of Gram-negative bacteria that are known to cause septic shock (6). However, nontoxic derivatives of LPS, such as monophosphoryl lipid A (MPL) and glucopyranosyl lipid A (GLA), are currently available for use either in experimental vaccines or approved to be used in human vaccines (92, 93). Pre-clinical trials in mice showed that GLA can function as a strong T$_h$1 adjuvant that can also stimulate potent cellular immune responses (94). Yet the only approved TLR4 agonist for human applications is MPL, which has been tested in many clinical trials as a cancer vaccine adjuvant (95). Several reports from the clinical trials suggested that MPL-adjuvanted vaccines are safe and immunogenic (72, 95).

TLR7 ligands. TLR7 recognizes viral single-strand RNA and imidazoquinoline derivatives, including resiquimod (R848) and imiquimod (6). Among the known TLR7 ligands, imiquimod, which was originally used as an anti-viral topical medication for the treatment of genital warts, is the only one that has been approved for the treatment of precancerous skin lesions on the skin (actinic keratosis) by the FDA (96). Although the mode of action is not completely understood, induction of type I interferons via the TLR7–MyD88–IRF7 pathway is known to play a major role for mediating its anti-viral and anti-tumor activities (66, 96). Moreover, an imiquimod-based liquid formulation, TMX-101 (Vesimune), is currently being tested in a phase II clinical trial for noninvasive bladder cancer patients as an immunotherapeutic agent (74).

TLR9 ligands. TLR9 is another endosomal TLR that recognizes intracellular DNA molecules of microbial origin because of the presence of unmethylated CpG motifs that are found in bacterial and viral genomes (6). CpG ODN is a synthetic TLR9 ligand capable of activating the TLR9–MyD88–IRF7 signaling pathway to induce type I interferons in addition to activating the TLR9–MyD88–NF-κB signaling pathway to induce pro-inflammatory cytokine production from immune cells (97).

Furthermore, several clinical trials that have been conducted in humans for evaluating the adjuvant activities of CpG ODNs demonstrated that CpG ODNs can induce T$_h$1-type immune responses, thereby becoming potential cancer vaccine adjuvants (97). Among the different types of CpG ODNs, D type ODN (also known as class A ODN) can potently induce type I interferon production from plasmacytoid DCs (pDCs) but fails to activate B cells for antibody production. However, because of the presence of poly-G tails, D type ODN can form aggregates, limiting its applications for clinical use.

On the other hand, K type CpG ODN (also known as class B ODN), such as K3 CpG, doesn't form aggregates in solution, and it is capable of potently activating B cells for antibody and IL-6 production while only weakly inducing type I interferon production from pDCs. Therefore, the clinically available CpG ODN is a K type ODN (97, 98). Clinical trials using CpG ODN as immunotherapeutic agents in cancer patients, such as melanoma and NSCLC, suggested that combination with chemotherapy or CpG ODN monotherapy can induce potent anti-tumor immune responses that correlate with clinical benefit (75, 99). Yet the potency of CpG ODN as a cancer vaccine adjuvant or anti-tumor agent needs further investigation.

Modified TLR9 ligands. Despite the promising immunotherapeutic potential of CpG ODNs in cancer immunotherapy, problems, such as inefficient cellular uptake and lack of cell-specific targeting, remain to be solved (100).

Therefore, several attempts have been made to enhance the efficacy of CpG ODNs either by using them in combination with other adjuvants or by chemically conjugating them to different nanoparticles or tumor-specific antigens in order to achieve optimal antigen/adjuvant delivery into APCs. Particularly, pre-clinical trials in mice demonstrated that compared with free CpG ODN, antigen- or nanoparticle-conjugated CpG ODN has the ability to induce more potent anti-tumor immune responses when used not only as an adjuvant but also as an anti-tumor agent (101–104). Moreover, Gungor *et al.* (105). recently developed novel CpG ODN nanorings, using the K type CpG ODN and the Tat peptide (47–57) of HIV. Their studies showed that because of enhanced adjuvant uptake and subsequent type I interferon production by pDCs, CpG ODN nanorings function as a potent T$_h$1 adjuvant that can also enhance anti-tumor immunity in mouse tumor models (105).

K3-schizophyllan. Another novel nanoparticle form of K type CpG ODN is K3-schizophyllan (K3-SPG) that was recently developed by our group by wrapping K3 CpG with the nonagonistic Dectin-1 ligand, SPG. *In vitro*, K3-SPG was able to activate human peripheral blood mononuclear cells for robust type I and type II interferon production. *In vivo*,

K3-SPG acted as a potent vaccine adjuvant with the ability to elicit robust antigen-specific cellular immunity, humoral immunity and, more importantly, CTL responses (77). Although its ability to function as an immunotherapeutic agent or adjuvant for cancer vaccines remains to be tested, because of the type of immune responses that are induced by K3-SPG, we hypothesize that it could be a useful adjuvant for cancer immunotherapy.

Hereby, we conclude that particulate forms of CpG ODN, such as K3-SPG, CpG ODN nanorings and CpG microparticles, are more efficacious therapeutic agents than free CpG ODN is (77, 105, 106), because nanoparticle forms of CpG ODN can achieve optimal adjuvant/antigen delivery to APCs or the tumor site to promote robust anti-tumor immune responses.

Combination adjuvants

TLR9 + STING ligands (K3 CpG + cGAMP)

Although K type CpG ODN (K3 CpG) is a clinically available T_h1 adjuvant, its therapeutic applications are limited, as it is a weak interferon inducer (97). On the other hand, cyclic dinucleotides, such as cGAMP, functioning as a second messenger in bacteria, have been reported to bind to the adaptor molecule STING and induce type I interferon production via the TBK1–IRF3 signaling pathway (107, 108).

Pre-clinical studies suggested that cyclic dinucleotides are potential vaccine adjuvants, eliciting antigen-specific B- and T-cell responses (58). Nevertheless, our group previously showed that activation of the STING–TBK1–IRF3 pathway by the STING ligand, DMXAA, causes induction of undesired type 2 immune responses (109). Therefore, therapeutic applications of STING ligands in cancer immunotherapy are also limited. Hence, to overcome these limitations, we combined the TLR9 ligand, K3 CpG, with the STING ligand, cGAMP, and found that together they act in synergy to induce innate and adaptive type II interferon (IFN-γ) resulting in potent type 1 adjuvants and anti-tumor agents. *In vitro*, the combination is capable of synergistically inducing IFN-γ production from NK cells via the concerted action of IL-12 and type I interferons (76).

Moreover, *in vivo*, we found that whereas cGAMP acts as a type 2 adjuvant potently inducing type 2 immune responses (IgG1 type antibody and IL-13 from T cells), the combination of cGAMP with K3 CpG almost completely suppresses these type 2 immune responses and makes a potent type 1 adjuvant capable of inducing robust type 1 immune (IgG2c type antibody and IFN-γ from T cells) and CTL responses. Furthermore, using mouse tumor models, we showed that the combination is also a powerful antigen-free anti-tumor agent that can induce the regression of B16 F10 and EG-7 tumors when injected intratumorally. In conclusion, combinatorial use of K3 CpG and cGAMP both as a type 1 adjuvant and as an anti-tumor agent is superior to their singular use (76).

Adjuvant Systems

Adjuvant Systems (GlaxoSmithKline) are different combinations of currently available vaccine adjuvants including alum, MPL and CpG ODN aiming at development of more efficacious vaccines capable of strongly eliciting antigen-specific immune responses especially in immunocompromised people (110). Adjuvant Systems that have already been licensed for human vaccines are AS03 (for influenza vaccines) and AS04 (for hepatitis B virus and HPV).

Besides, several Adjuvant Systems are currently being tested in pre-clinical or clinical trials for cancer (111). For instance, AS02, composed of MPL, QS-21 and oil-in-water emulsions, has been tested in patients with MAGE-A3+ tumors, such as melanoma and NSCLC, as a cancer vaccine adjuvant. These clinical trials revealed that AS02 is able to induce MAGE-A3-specific antibody and T-cell responses, which may provide benefit to the patients (78, 112).

Furthermore, AS15, composed of CpG ODN, MPL, QS-21 and liposomes, has been reported to be immunogenic (79), and it was used in phase II and III clinical trials involving patients with melanoma or NSCLC (111). Although phase II trials in melanoma patients showed that AS15-adjuvanted vaccine was inducing protective anti-tumor immune responses and well tolerated, it failed to increase disease-free survival in the phase III clinical trial with NSCLC patients (113). Therefore, the immunotherapeutic potential of AS15 should be tested in cancers other than NSCLC.

Conclusions

In this review, we summarized recent progress in the development of novel adjuvants for cancer immunotherapy and the current clinical status of these adjuvants. Adjuvants are crucial components of vaccines for both infectious diseases and cancer as they can further promote the immune responses elicited to vaccines. In addition to being used as a part of vaccine, certain adjuvants, such as CpG ODN, can also serve as anti-tumor agents that can be used in cancer immunotherapy (97).

Mechanisms by which adjuvants can promote anti-tumor immunity generally rely on the stimulation of innate immunity via the PRRs, such as TLRs, upon which innate immune cells prime robust and sustained adaptive immune responses against the tumors. On the other hand, certain adjuvants, such as ISCOMs and nanoparticle forms of CpG ODN, can achieve potent anti-tumor immune responses by efficiently delivering the antigen and/or adjuvant into the tumor site or APCs. Moreover, several animal studies as well as clinical trials suggested that combination adjuvants, such as K3 CpG + cGAMP and adjuvant systems (e.g. AS02), capable of activating multiple PRRs and/or acting as efficient delivery systems, have higher efficacy than single adjuvants (76, 111).

Thus, more efficacious cancer vaccine adjuvants can be developed both by developing totally new compounds with adjuvant activities and by optimizing the adjuvant formulations using various combinations of well-known adjuvants. Furthermore, despite the promising results from clinical trials using emerging adjuvants, there are only a few adjuvants licensed for use in cancer immunotherapy, indicating the need for large-scale clinical trials and the necessity to characterize the types of immune responses elicited by these novel adjuvants as they can induce both tumor-supportive and anti-tumor immune responses.

As the information on mode of action and safety of the emerging adjuvants expands, we suppose that the number

of licensed adjuvants for cancer immunotherapy will gradually increase.

Funding

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