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Toll-like receptor agonists in cancer therapy

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Abstract

Toll-like receptors (TLRs) are pattern-recognition receptors related to the *Drosophila* Toll protein. TLR activation alerts the immune system to microbial products and initiates innate and adaptive immune responses. The naturally powerful immunostimulatory property of TLR agonists can be exploited for active immunotherapy against cancer. Antitumor activity has been demonstrated in several cancers, and TLR agonists are now undergoing extensive clinical investigation. This review discusses recent advances in the field and highlights potential opportunities for the clinical development of TLR agonists as single agent immunomodulators, vaccine adjuvants and in combination with conventional cancer therapies.

Keywords

bacillus Calmette–Guerin; CpG; imiquimod; immunotherapy; monophosphoryl lipid A; polyriboinosinic-polyribocytidylic acid; resiquimod; Toll-like receptor; Toll-like receptor agonist; vaccine adjuvant

Background

The innate immune system utilizes a variety of transmembrane or secreted pattern-recognition receptors (PRRs), which are vital for activation of complement and coagulation cascades, opsonization, phagocytosis, apoptosis and induction of proinflammatory mediators. Toll-like receptors (TLRs) are important members of PRRs, initially discovered as Toll involved in *Drosophila* dorsoventral embryonic development and antifungal immunity [1]. In 1997, Medzhitov *et al.* cloned a human homolog of the *Drosophila* Toll protein, now known as TLR4, and showed that the Toll/NF-κB signaling pathway is conserved in humans, and that Toll signaling stimulated adaptive immune responses [2]. Shortly after, TLR4 was shown to be involved in the recognition of lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria, which established the link between mammalian TLRs and recognition of pathogen-associated molecular patterns (PAMPs) [3–5]. PAMPs are conserved motifs shared by many bacteria, viruses, protozoa and fungi; examples are LPS and lipoteichoic acid (all

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recognized by TLR4); peptidoglycans (cell walls), lipoproteins (bacterial capsules) and zymosan (all recognized by TLR2 following heterodimerization with TLR1 or TLR6); flagellin (in bacterial flagella, recognized by TLR5), unmethylated bacterial or viral CpG DNA (recognized by TLR9) and viral RNA (single-stranded recognized by TLR7 and TLR8, double-stranded by TLR3).

Toll-like receptors belong to the TLR-IL-1 receptor (TIR) superfamily; they share an ectodomain composed of leucine-rich repeats for recognition of PAMPs and an intracytoplasmic TIR domain that mediates the recruitment of adapter molecules such as myeloid differentiation factor-88 (MyD88), TIR-associated protein (TIRAP), Toll receptor-associated-activator of interferon (TRIF) and/or Toll-receptor-associated molecule (TRAM). While most TLRs are expressed on the cell surface, TLR3, 7, 8 and 9 are found within endosomes, where they are activated following capture and internalization of pathogens or their products [6,7]. Some cell-surface TLRs are internalized after ligand binding; TLR2, for example, is recruited to macrophage phagosomes [8].

Toll-like receptor signaling is detailed in Figure 1; pathway activation results in transcription of type I IFN genes and proinflammatory cytokine genes such as *TNF*-α, *IL-1* and *IL-6* [6]. The cytokine induction pattern is determined by the type of TLR-activated cell. Stimulation of human TLR7, for instance, induces IFN-α from plasmacytoid dendritic cells (pDCs) important for innate antiviral immunity and the development of adaptive immunity, whereas it induces IL-12 from myeloid dendritic cells (mDCs), associated with the induction of a Th1 response [9]. It has also been suggested that, at least for murine TLR4, relative activation of its two distinct downstream signaling pathways affects the therapeutic index of the agonist [10]. Despite differences in the induced cytokine pattern defined by dendritic cell (DC) subset, TLR agonist and signaling adaptors, TLR activation generally results in activation and phenotypic maturation of all DCs.

Toll-like receptors are invariant receptors, preferentially expressed in cells of the innate immune system. They provide an organism-wide sensing system; therefore, the expression pattern is aligned with the cell's frontline effector role in host defense. The expression and regulation of TLR genes in humans differ from those of other species, for instance mice [11], placing important limitations on the interpretation of animal models. In humans, ten distinct TLRs have been described to date. mDCs express TLR1–6 and 8 whereas pDCs express TLR7 and 9 [9,12–15]. TLR7 expression remains controversial in mDCs and monocyte-derived DCs (moDCs) [12,14]. Neutrophils express TLR1, 2 and 4–10, natural killer (NK) cells express TLR1, monocytes express all TLRs except TLR3 and B lymphocytes express TLR9 and 10 [16–19]. Activated T-cell subsets; including memory cells, express TLR2 as a costimulatory receptor [20], whereas regulatory T cells (Tregs) can express TLR8 and 10 [21,22]. While TLRs are primarily expressed in hematopoetic cells, they have also been described in keratinocytes [23] and epithelial cells of the intestinal, urogenital and respiratory tracts [24–26], and are likely to provide antimicrobial defense in addition to the mechanical barrier of the epithelial layer.

The importance of TLRs in the host response to tumors is evident from the fact that a link between polymorphisms in TLR genes has been established not only for susceptibility to infections but also for cancers [27]. For instance, carriers of the *TLR2* rs3804100 variant are at greater risk for developing marginal zone lymphoma (MZL) [28]. The association between *TLR2* and MZL is conceivable given the strong evidence linking *Helicobacter pylori* to the pathogenesis of mucosa-associated lymphoid tissue lymphoma, a common subtype of MZL, and the role of TLR2-modulated immune responses to this pathogen [29,30].

Rationale for TLR agonists as cancer therapeutics

Systemic antitumor effects of streptococcal infections were reported over a century ago [31]. These observations were followed by attempts to treat cancer with Coley's toxin (a mixture of heat-killed *Streptococcus pyogenes* and *Serratia marcescens*), which cured a subset of patients with inoperable sarcoma [32]. In retrospect, antitumor effects were probably due to TLR-activation by endotoxins and unmethylated sequences in bacterial DNA.

TLR3, 4, 7/8 and 9 agonists represent promising cancer immunotherapeutics and have been included in the ranked National Cancer Institute's list of immunotherapeutic agents with the highest potential to treat cancer [201]. They provide essential requirements for initiating T-cell immunity: antigen uptake, processing and presentation by DCs and other APCs, DC maturation and T-cell activation. TLR-mediated DC activation leads to enhanced phagocytosis, maturation with upregulation of MHC and costimulatory molecules (CD80, CD86 and CD40), upregulation of CCR7 and migration to draining lymph nodes, secretion of directive cytokines and antigen presentation to lymphocytes [15]. Type I IFNs are the most prevalent cytokines induced upon TLR3, 7, 8 and 9 activation. While often associated with antiviral defense, type I IFNs are also essential for several aspects of the adaptive immune response including facilitation of antigen cross-presentation, proliferation of memory T cells, prevention of T-cell apoptosis, induction of DC maturation and activation of NK cells [15]. Of particular interest to the development of TLR agonists for anticancer therapy are the effects of the TLR-induced cytokines IL-6 and IL-12 [33,34]. IL-6 can enhance antigen-specific T-cell activation through suppression of Tregs; IL-12 skews the effector response toward a Th1 profile [35], although IL-12-independent pathways for Th1 differentiation have been recently described after TLR4 stimulation [36]. TLR7/8 activation by imidazoquinolines can also enhance antitumor effects through inhibition of angiogenesis, NK-mediated cytotoxicity and direct apoptosis of tumor cells [37–39]. Furthermore, local TLR7 activation by imiquimod has been shown to alter the tumor microenvironment and create an inflammatory environment suitable for antigen cross-presentation and infiltration by effector T cells and DCs with cytotoxic potential [40].

Toll-like receptor agonists undergoing clinical investigation for cancer are reviewed in the following section and include live or killed bacteria, viral agents and synthetic compounds (Table 1).

TLR agonists as monotherapy for cancer

Toll-like receptor agonists used as single agents especially when applied locally can effectively eradicate tumors due to their potent stimulation of innate and adaptive immunity as well as their effects on the tumor microenvironment (Figure 2). Two TLR agonists, bacillus Calmette–Guerin (BCG) and imiquimod are US FDA approved for clinical use as monotherapy for cancer.

Bacillus Calmette–Guerin

A live, attenuated *Mycobacterium bovis* prepartaion of bacillus of the Calmette–Guerin strain (TICE™ and PACIS™) stimulates TLR2 and TLR4 via its mycobacterial components, including cell wall skeleton and peptidoglycan, as well as TLR9 via its bacterial DNA [41–45]. BCG is approved for intravesical therapy of bladder carcinoma *in situ* and superficial bladder cancers. Initially tested by local injections into melanoma metastases, it was observed that inflammation was accompanied by tumor regression in skin or urinary bladder [46,47]. Extensive clinical investigation of BCG in patients with bladder cancer showed antitumor activity in the majority of patients. Compared to intravesical doxorubicin chemotherapy, BCG led to greater complete response rates for Tis lesions (70 vs 34%; $p < 0.001$) and improved 5-year disease-free survival in patients with Ta/T1 tumors (37 vs 17%; $p = 0.015$) as well as Tis

tumors (45 vs 18%; p = 0.01) [48]. BCG's antitumor activity has been linked to cytokines locally secreted by the host, especially IFN-γ and IL-2 [49–51] and the development of antigen-specific T-cell immunity [52]. While BCG is typically well tolerated, with only mild to moderate side effects (mainly cystitis and influenza-like symptoms), serious infections can occur that may require antituberculous treatment [53]. BCG's regulatory approval marked the first compound (pathogen) approved for the treatment of cancer, which primarily acts via TLR stimulation.

Imiquimod

Imiquimod is a synthetic imidazoquinoline that targets TLR7. Formulated as imiquimod 5% cream (Aldara™), it is indicated for the treatment of a variety of diseases ranging from human papillomavirus (HPV)-related external genital and perianal warts (condylomata acuminata) to primary skin malignancies (superficial basal cell carcinoma [sBCC]) or premalignant conditions (actinic keratosis). Treatment of sBCC with imiquimod 5% cream resulted in histologic clearance rates of 79–82% in Phase III studies [54]. Objective responses have also been observed in selected melanoma patients when imiquimod was directly applied onto cutaneous metastases [55,56]. A Phase II study of imiquimod in breast cancer patients with chest wall recurrences or skin metastases is ongoing [202]. Imiquimod's antineoplastic effects result from the induction of innate and adaptive immunity, reversal of local tumor-induced immunosuppression and induction of tumor-cell specific apoptosis [39,57–60]. Topical use of imiquimod cream has shown an excellent safety profile with reversible local reactions and minimal systemic exposure [61,62]. Orally administered imiquimod induced systemic IFN-α in patients with advanced cancers, but tumor responses were not observed as immune-related side effects limited dosing [63]. A newer imidazoquinoline TLR7 agonist, 852A, administered parenterally as monotherapy has shown only modest clinical efficacy with disease stabilization observed in some patients in Phase I and II studies of advanced cancer [64,65], raising again the possibility that systemic side effects preclude the evaluation of greater doses.

While not yet investigated for the topical treatment of invasive cancers, resiquimod, an imidazoquinoline TLR7/8 agonist in humans, holds great promise as a single agent or vaccine adjuvant [66,67]. In addition to TLR7-induced IFN-α production by pDCs, TLR8 activation of human monocytes and mDCs leads to the secretion of proinflammatory cytokines and chemokines such as TNF, IL-12 and macrophage inflammatory protein (MIP)-1 [68]. The induction of IL-12, enhancement of NK-cell cytotoxicity and TLR8-mediated suppression of Tregs are particularly desirable features for cancer immunotherapeutics [21,68,69]. When studied in patients with actinic keratosis, topical treatment with resiquimod resulted in 77–90% complete clearance of lesions [70].

CpG oligodeoxynucleotides

CpG are single-strand oligodeoxynucleotides (ODNs), characterized by motifs containing cytosines and guanines. Based on their immunologic effects, CpG ODNs are divided into three different classes: CpG-A, a potent stimulator of NK cells owing to its IFN-α-producing effect on pDCs; CpG-B, a moderate IFN-α inducer, and enhancer of antigen-specific immune responses (upregulates costimulatory molecules on pDCs and B cells, induces Th1 cytokine production and stimulates antigen presentation by pDCs); and CpG-C, which combines the stimulatory capacity of both CpG-A and CpG-B [71,72].

CpG 7909 (PF-3512676, a CpG type B and TLR9 agonist) has been evaluated in several tumor types including renal cell carcinoma, glioblastoma, melanoma, cutaneous T-cell lymphoma and non-Hodgkin's lymphoma [73]. A 10% response rate in patients with metastatic melanoma was observed, along with evidence of immune activation in a Phase II trial of CpG given as subcutaneous injection [74]. Intralesional CpG showed antitumor activity against basal cell

carcinoma and melanoma in a Phase I study [75] in the setting of local and systemic immune activation. When injected intradermally around primary melanoma excision sites CpG resulted in pDC and mDC activation, a Th1 cytokine profile, and reduced Treg frequencies in the draining lymph node [76], augmenting melanoma-antigen specific T lymphocytes and NK-cell responses [77].

Polyriboinosinic-polyribocytidylic acid

Polyriboinosinic-polyribocytidylic acid (Poly I:C) is a synthetic analog of viral dsRNA that stimulates endosomal (TLR3) and/or cytosolic melanoma differentiation-associated gene 5 (*MDA5*), leading to increased production of type I IFNs [78,79]. Poly I:C and its more stable cousin Poly I:C-poly-L-lysine (Poly ICLC) were evaluated as single agents in cancer patients several decades ago. Intravenous or intramuscular administration of Poly I:C showed no clinical activity in several cancers [80], probably due to its rapid degradation in human plasma [81]. The more stable compound Poly ICLC did induce systemic IFNs; however, dosing was limited by constitutional symptoms and no antitumor efficacy was seen in patients with metastatic melanoma [82]. Single-agent treatment of recurrent anaplastic glioma with Poly ICLC at lower doses to reduce toxicity did not translate into clinical benefit when compared with historical controls [83]; a lack of benefit with low-dose Poly ICLC was also observed in renal cell carcinoma [84].

In summary, TLR ligands are successfully and safely used as monotherapy for some cancers when applied locally (intravesical and percutaneous), but their systemic use remains limited because immunostimulatory effects required for antitumor efficacy come at the expense of systemic toxicity.

TLR agonists as adjuvants for cancer vaccines

Toll-like receptor agonists represent promising vaccine adjuvants as they are potent DC activators, augment T-cell responses and downregulate suppressive effects of Tregs. The following section focuses on the clinical development of TLR agonists as vaccine adjuvants for cancer therapy. Many infectious disease vaccines, both licensed and experimental, engage TLRs through intrinsic or added adjuvants, a topic that has been reviewed elsewhere [85,86].

Bacillus Calmette–Guerin

Bacillus of the Calmette–Guerin adjuvant has been extensively evaluated with cellular vaccines in colorectal cancer (CRC) and malignant melanoma [87,88]. Despite promising Phase II data, the randomized Phase III trial of BCG administered with an allogeneic melanoma vaccine (Canvaxin™) failed to improve disease-free and overall survival compared to BCG alone in patients with resected stage III and IV melanoma. Better outcomes were instead reported for the BCG alone arm [87]. The reason for this unexpected observation remains unclear, though it is possible that antigen-specific immune responses may have been suppressed by the mixture of irradiated melanoma lines.

Clinical benefit, along with an excellent safety profile, was observed in a placebo-controlled, randomized Phase III trial after vaccinating CRC patients with autologous tumor cells and BCG in the adjuvant setting [88]. The vaccine arm showed significantly reduced recurrence rates (44%; $p = 0.023$) and improved recurrence-free survival (42%; $p = 0.032$) for patients with stage II disease, leading to regulatory approval in The Netherlands. However, no benefit was seen in stage III patients.

Bacillus Calmette–Guerin followed by granulocyte–macrophage colony-stimulating factor (GM-CSF) as vaccine adjuvant for NY-ESO-1 protein induced antigen-specific humoral and

cellular immune responses in a single-arm pilot study of patients with urothelial carcinoma [89].

Monophosphoryl lipid A

Lipid A molecules that target the TLR4 complex are among the most commonly used vaccine adjuvants. One promising candidate is monophosphoryl lipid A (MPL), a derivative of lipid A from *Salmonella minnesota*. It was identified in the 1980s through systematic modification of LPS (the prototype TLR4 agonist) of *S. minnesota*, lacking LPS toxicity in humans. MPL adjuvant has shown an excellent safety and efficacy profile in several thousands of patients, received regulatory approval in Europe as adjuvant for a hepatitis B vaccine (Fendrix™) and is used as adjuvant in a HPV vaccine (Cervarix™) for which a Biologics License Application has been filed with the US FDA.

DETOX adjuvant, which contains MPL and cell wall skeletons of *Mycobacterium phlei*, has been evaluated in Phase I/II vaccine studies in several cancers. In melanoma patients, cellular immune responses to vaccination with a combination of melanoma cell lysates (Melacine™), or irradiated autologous tumor cells with DETOX, correlated with clinical responses [90,91]. Synthetic sialyl-Tn haptens conjugated to keyhole limpet hemocyanin (STn-KLH, Theratope™), together with DETOX adjuvant, have been used in pilot vaccination studies for mucin-expressing epithelial tumors, including breast cancers, and have shown safety and occasional tumor regressions [92]. Vaccination after high dose chemotherapy and autologous stem cell rescue resulted in humoral and cellular immune responses; however, no clinical benefit was demonstrated with this approach [93,94]. Immune responses were also demonstrated in a Phase I study of mutated ras peptides combined with DETOX [95].

Monophosphoryl lipid A adjuvant is also a component of the liposomal Stimuvax® vaccine targeting MUC-1 in advanced non-small-cell lung cancer (NSCLC), and is currently undergoing Phase III evaluation worldwide. The Phase II study suggested clinical benefit in NSCLC stage IIIb patients in the absence of a malignant pleural effusion [96].

Monophosphoryl lipid A is also used as part of a proprietary immunological adjuvant system (AS15, AS02b) in a MAGE A3 vaccine against MAGE A3-expressing NSCLC and malignant melanoma [97]. AS15 is a liposomal formulation of CpG, MPL and QS21 (a natural saponin molecule extracted from the bark of the South American tree *Quillaja saponaria molina*). AS02b consists of MPL and QS21 in an oil-in-water emulsion. A placebo-controlled, randomized Phase II trial of MAGE A3/AS02b confirmed an excellent safety profile and suggested clinical benefit with prolonged disease-free survival for patients with resected stage I and II NSCLC (HR = 0.73; 95% CI: 0.45– 41.16) [98], leading to the initiation of a global Phase III trial of MAGE A3/AS15 in patients with resected stage Ib–IIIa NSCLC. The Phase II trial in patients with metastatic melanoma confirmed a safe vaccination approach and showed superiority of adjuvant AS15 over AS02b suggesting that the addition of TLR9 agonist CpG to MPL increased vaccine efficacy [99]. A follow-up Phase III study of MAGE A3/AS15 is ongoing for patients with resected, high-risk melanoma.

CpG oligodeoxynucleotides

CpG ODNs, short DNA sequences containing unmethylated CpG motifs stimulating TLR9, have been used successfully as adjuvants in cancer vaccines when added to cancer-testis antigens emulsifed in Montanide ISA 51 [100,101]. While studies lacked a concurrent non-CpG control arm, comparisons with vaccines containing antigen/Montanide ISA 51 are suggestive of substantially increased immunogenicity of the CpG-containing formulation [100]. The vaccine combination with antigen and/or emulsification in Montanide ISA 51 may be necessary for CpG's adjuvant effect, as CpG did not induce antigen-specific immunity in

patients with advanced cancers when given sequentially after GM-CSF before antigen injection [102]. Interestingly, CpG induced profound transient peripheral lymphopenia that coincided with lymphocyte infiltration at the vaccination site, probably due to pDC-derived chemoattractants. Intratumoral injection of CpG may therefore be a promising strategy to direct the effector response after vaccination. CpG is also part of the adjuvant system AS15 discussed above, which is being evaluated in two Phase III MAGE A3 vaccination trials.

Imiquimod

Imiquimod, a synthetic imidazoquinoline that targets TLR7 has demonstrated immunomodulating effects as a topical vaccine adjuvant [103,104]. Injection of immature DCs into imiquimod-pretreated skin led to DC activation and migration to draining lymph nodes in cancer patients, as well as to enhanced DC immunostimulatory capacity [105]. Topical imiquimod treatment augmented the immunogenicity of a melanoma peptide vaccine when administered with systemic Flt-3 ligand [103]. Our group recently demonstrated imiquimod's adjuvant activity when administered topically with NY-ESO-1 protein vaccination in patients with resected melanoma. Imiquimod induced recruitment and activation of mDCs and pDCs in the treated skin, as well as inflammatory infiltrates (Figure 3). The vaccine combination showed an excellent safety profile, elicited humoral and cellular NY-ESO-1-specific immune responses in a significant fraction of patients and was easy to administer [104]. Indoleamine 2′3′-dioxygenase (IDO), an enzyme involved in catabolism of tryptophan and generation of kynurenine metabolites that results in suppression of activated T cells and induction of functional Tregs after TLR7 activation of human pDCs [106], was not detected in our study. A follow-up study of topical resiquimod, a TLR7/8 agonist, in combination with a NY-ESO-1 protein in Montanide ISA 51 emulsion, is ongoing.

Polyriboinosinic-polyribocytidylic acid

Polyriboinosinic-polyribocytidylic acid and derivatives are being studied as vaccine adjuvants in cancer trials, as potent DC maturation and induction of a Th1 cytokine profile has been demonstrated *in vitro* [107], and preclinical studies have shown promising results [108]. Depending upon formulation and delivery, endosomal (TLR3) and/or cytoplasmic (MDA5) pathways are differentially accessed, as recently shown in preclinical models [109]. Several studies of Poly ICLC administered with peptide or DC vaccines are ongoing in patients with various advanced malignancies, including malignant glioma [202]. Efficacy data have not yet been reported.

Manipulation of TLR to improve the immunogenicity of *ex vivo* prepared DC vaccines

We have recently shown that mDCs matured *ex vivo* with a cytokine cocktail (IL-1β, IL-6, TNF-α and PGE2) are suboptimal in inducing cellular immune responses in cancer patients when compared with Montanide, a commonly used vaccine adjuvant [110]. *Ex vivo* TLR-activated DCs or genetically modified DCs expressing TLR-stimulating receptors may be superior for immunization protocols. Preliminary clinical data are promising; human DCs electroporated with constitutively active TLR4-encoding mRNA are potent T-cell stimulators [111] and Poly I:C-activated DCs produced high amounts of the Th1 cytokine IL-12p70 [112]. LPS-activated DCs pulsed with Her2 peptides injected intranodally prior to surgical resection of $Her2^{+}$ ductal carcinoma *in situ* resulted in the regression of tumors, accompanied by the induction of Her2-specific cellular immune responses, loss of Her2 expression and lymphocytic infiltration in the tumor [113].

TLR modulation by chemotherapy & radiation

Recently demonstrated in mice, TLR4 signaling in DCs triggered by chemo- and radiotherapy-induced cell death contributes to the generation of an antitumor immune response [114]. Furthermore, TLR4 and its activation were required for the efficacy of radiation and chemotherapy. These findings are consistent with clinical observations that TLR4 polymorphism (TLR4 Asp299Gly) is associated with worse outcomes in patients with operable breast cancers receiving adjuvant anthracycline-based chemotherapy [114].

Lymphodepleting preparative regimens employed prior to adoptive T-cell transfer in tumor-bearing mice can also activate the innate immune system via TLR4, as total-body irradiation or chemotherapy-induced gut injury permitted translocation of microbes [115,116]. TLR4 activation by bacterial LPS was one of several mandatory factors contributing to the effectiveness of adoptively transferred cells; therefore, manipulation of TLRs could be employed to further enhance adoptive immunotherapy for cancer. In addition, the understanding of genetic variations of TLR polymorphisms and TLR activation in response to conventional cancer therapies will greatly enhance our ability to tailor treatments to the individual patient.

Combination of TLR agonists & conventional cancer therapies

Promising areas for TLR agonists are their use in combination with conventional cancer treatments to enhance cytotoxic chemotherapies, ionizing radiation or monoclonal antibodies. TLR activation enhances the immune response to antigens released, potentially resulting in therapeutic synergy with traditional cancer treatments. Additional benefits of chemotherapy, ionizing radiation and monoclonal antibodies are the reduction of tumor bulk (leading to a smaller target for the immune effectors as well as decreased tumor-associated immunosuppression), upregulation of MHC expression necessary for effective immunity, sensitization of tumor cells to immune destruction via upregulation of FasL and removal of immunosuppressive Tregs [117–120]. In addition, cytotoxic cancer therapies can release molecules such as high-mobility group box 1 protein (HMGB1) and heat-shock proteins, which serve as endogenous ligands for TLR2 and/or TLR4. Clinical relevance of TLR4 modulation by endogenous ligands has been demonstrated in breast cancer, as discussed above [114]. Furthermore, HMGB1 released from necrotic cells can also modulate pDC and B-cell function in response to DNA in a TLR9-dependent manner [121–123].

TLR2/4 agonists also enhance the effect of chemotherapy via TNF-α and inducible nitric oxide synthase (iNOS)-dependent tumor cell apoptosis in animal models [124].

In addition, TLR2/4 and 7 agonists inhibit tumor-associated angiogenesis [38,124]. The restoration of ‘normal’ vasculature decreases interstitial pressure that allows efficient drug delivery into the tumor and possibly decreases metastases [125].

Synergy of TLR agonists with chemotherapeutic approaches, radiotherapy and monoclonal antibodies has been successfully demonstrated in murine models of sarcoma, mammary carcinoma and lymphoma [126–129]. Pilot studies in patients with malignant glioma and lymphoma support the notion of synergistic effects of TLR9 agonist CpG with chemotherapy, radiation or monoclonal antibodies (rituximab) and showed clinical benefit and an excellent safety profile [130–132].

In a randomized Phase II study, the addition of TLR9 agonist PF-3512676 (CpG 7909, which belongs to the CpG-B class) to first-line treatment with platinum-based chemotherapy in stage IIIb–IV NSCLC improved the overall response, and suggested a trend toward improved survival (12.3 vs 6.8 months; $p = 0.18$) [133]. The confirmatory Phase III trial was discontinued

at the scheduled interim analyses, as the chemotherapy/PF-3512676 combination did not result in clinical benefit for this population [134]. Failure of this approach may be owing to a lack of 'context' as CpG 7909 was delivered distant from the tumor as subcutaneous injection. Furthermore, NSCLC is a relatively chemotherapy resistant malignancy, possibly compromising the availability of tumor antigens; therefore, this combinatorial approach may be more effective in malignancies such as ovarian cancer which are highly sensitive to front-line cytotoxics. It is also possible that the chemotherapeutic regimen (taxane and platinum) interfered with the development of an antigen-specific immune response or antagonized the effects of the CpG, possibly at the MyD88 level as paclitaxel can recruit MyD88 as a TLR4 agonist [135] as well as in a TLR-independent manner [136].

Intratumoral injections of CpG after local radiation is currently under clinical investigation in patients with B-cell lymphoma [202]. This approach is designed to activate intratumoral DCs to facilitate antigen presentation after radiation-induced apoptosis of the tumor [120]. CpG is also being studied in combination with monoclonal antibodies such as anti-CD20 (rituximab), as CpG ODN have been shown to upregulate CD20 on human malignant B cells [137] and increase the expression of several IFN-inducible genes [132]. A Phase II study confirmed the safety of this approach, and while the contribution of CpG to clinical and immune responses can only be determined in a randomized, controlled trial, significant $CD8^+$ T-cell infiltration observed in the tumor after treatment is suggestive of an enhanced immune response [138].

Conclusion & future perspective

Toll-like receptor agonists possess important properties that can be exploited for the immunotherapy against tumors. Their ability to promote tumor-specific Th1 and cytotoxic T lymphocyte responses has translated into antitumor activity in many preclinical and clinical studies, leading to the approval of two TLR agonists for cancer therapy when applied locally to tumors. However, systemic use of TLR agonists may be restricted by dose-limiting toxicities owing to systemic cytokine induction.

Combinatorial approaches of TLR agonists with conventional therapies such as chemotherapy or radiation are promising, as dosing requirements may be lower, and one can selectively target several pathways. In addition, the recent discovery that the therapeutic effects of conventional cancer treatments such as anthracyclines and radiation depend upon intact TLR signaling will greatly enhance our ability to test novel combinations, as well as to use genetic screening for TLR polymorphisms to guide the selection of individual treatments.

The use of TLR agonists as cancer vaccine adjuvants remains a promising area of investigation. New approaches conjugate the TLR agonist to the antigen of interest or utilize TLR agonist combinations as synergism have been demonstrated for several agonists [139,140]. As discussed above, two international Phase III studies are currently evaluating the clinical efficacy of adjuvant AS15, which contains a combination of TLR4 and 9 agonists, in MAGE A3 vaccination. Finally, combinations of TLR agonists with anti-CTLA4 antibody or targeted therapies may prove advantageous. Inhibition of p38 in DCs for instance, has been shown to attenuate TLR-induced IL-10 thus selectively suppressing the induction of Tregs as well as to augment TLR-induced IL-12 production critical for Th1 polarization [141]. Consistent with these observations, p38 inhibition enhanced the antitumor efficacy of TLR9-activated DCs in B16 murine melanoma [141].

It is important to carefully choose the agonist as TLR ligation could potentially trigger autoimmune diseases or severe inflammation, which have recently emerged as side effects of immunotherapies such as CTLA-4 blockade including dermatitis, colitis, hepatitis and hypophysitis [142]. Experimental asthma for instance is exacerbated by TLR2 agonists through

the induction of a Th2 profile in the setting of vaccination, whereas TLR9 agonists confer protection [143].

Executive summary

Toll-like receptors & their activation

- Toll-like receptors (TLR) recognize pathogen-associated molecular patterns, which are conserved motifs shared by bacteria, viruses, protozoa and fungi.
- TLR signaling activates innate and adaptive immune responses. TLR activation results in transcription of type I IFN and proinflammatory cytokine genes. The cytokine induction pattern is determined by dendritic cell (DC) subset, TLR agonist and signaling adaptors. TLR-mediated DC activation leads to enhanced phagocytosis, maturation and migration to draining lymph nodes, secretion of T helper cell-1 cytokines and antigen presentation to lymphocytes. Type I IFNs support an antitumor immune response by facilitating antigen cross-presentation, T-cell proliferation, DC maturation and natural killer cell activation.

TLR agonists as cancer therapeutics

- Synthetic agonists, microbial products and endogenous ligands can modulate TLRs.
- TLR agonists represent promising cancer immunotherapeutics, agents undergoing clinical investigation are reviewed in Table 1.
- Locally applied TLR agonists can effectively eradicate tumors owing to the induction of innate and adaptive immunity as well as their multiple effects on the tumor microenvironment.
- TLR agonists are also potent vaccine adjuvants as they activate DCs, augment T-cell responses and downregulate suppressive effects of regulatory T cells. While cancer vaccines remain investigational, many infectious disease vaccines, both licensed and experimental, engage TLRs through intrinsic or added adjuvants.
- Combinations of TLR agonists with chemotherapy, ionizing radiation, monoclonal antibodies and targeted therapies are being explored.

TLRs are modulated by chemotherapy & radiation

- TLR signaling can be triggered by chemotherapy- and radiotherapy-induced cell death and contributes to an antitumor immune response.
- TLR polymorphisms can affect outcomes in patients treated with conventional cancer therapies.

Conclusion

- Several TLR agonists possess important properties that can be exploited for the immunotherapy against tumors.
- The understanding of genetic variations of TLR polymorphisms and TLR activation in response to cancer therapies will greatly enhance the ability to tailor treatments to the individual patient.

The successful clinical development of TLR agonists requires careful selection and study of the agent as activation of some TLRs, albeit through tumor-derived soluble mediators, has been shown to enhance tumor growth and metastasis [144,145]. Furthermore, functional TLRs can be expressed by human tumors that may promote tumor progression, confer resistance to

apoptosis as well as suppress antitumor immune responses, leading to immune evasion [146, 147]. In contrast, TLR-induced apoptosis of several human cancers has been demonstrated, for instance treatment of cutaneous melanoma metastases with 5% imiquimod cream induced tumor-cell death in the majority of biopsied lesions [148]. TLRs may not be required for this process, although Poly I:C-triggered apoptosis of breast cancer *in vitro* is mediated through TLR3 expressed by tumor cells [149].

Coinduction or promotion of Tregs that may attenuate antitumor effects is also of concern for use of TLR agonists in cancer immunotherapy (reviewed in [150]). Strategies to limit Treg-mediated suppression may include p38 inhibition as described above [141] and the selective use of TLR4, 8 or 9 agonists. In murine models for instance, stimulation of TLR4 and TLR9 on DCs has been demonstrated to overcome Treg-mediated suppression of effector cells [33, 151]. TLR8 activation of human Tregs has been shown to reverse their function *in vitro*, and adoptive transfer of TLR8-stimulated Tregs into tumor-bearing mice enhanced antitumor immunity [21]. TLR8 manipulation is a particularly appealing approach for clinical studies as human Tregs express relatively high levels of TLR8.

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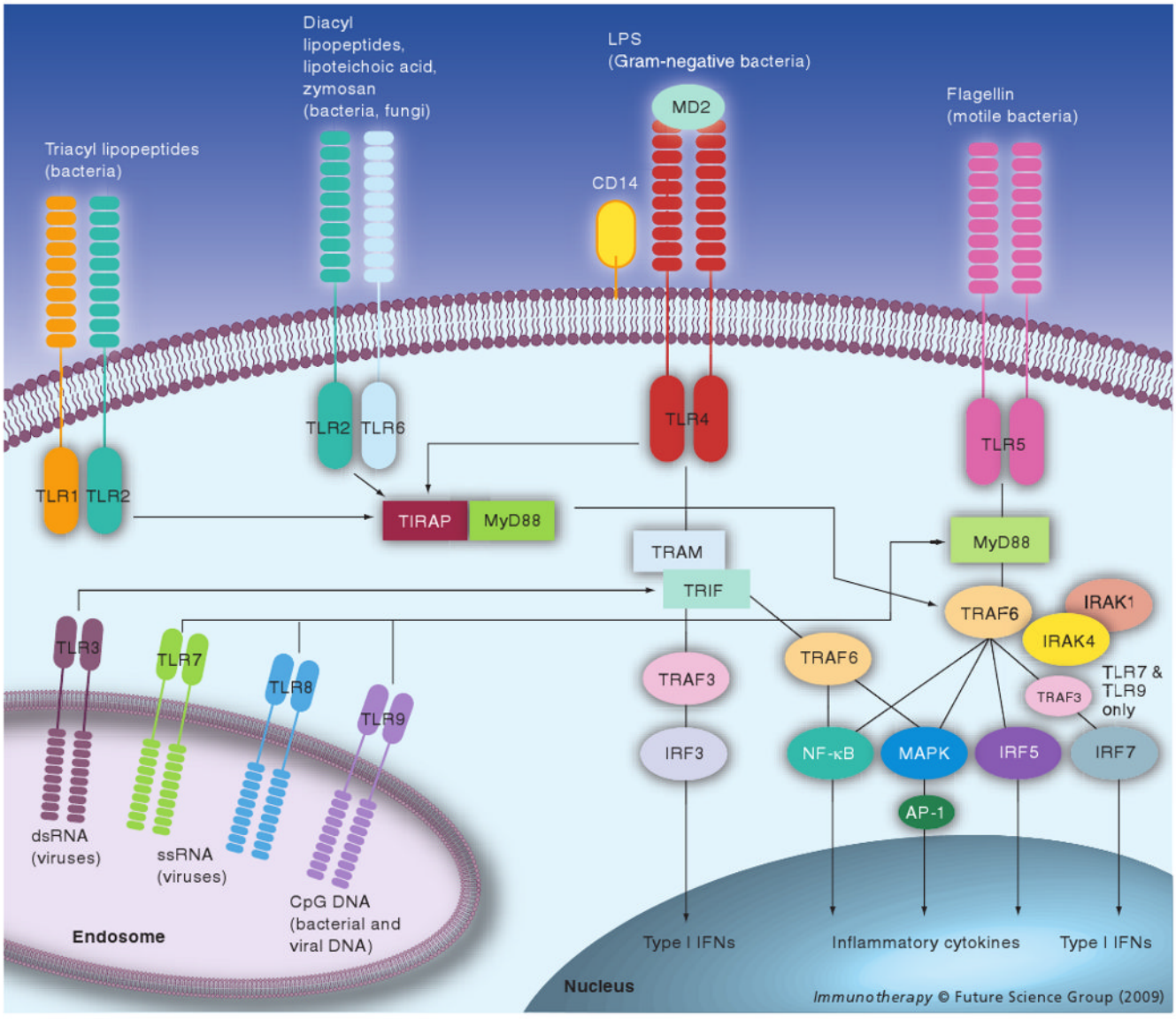

Figure 1. Toll-like receptor signaling

Toll-like receptors (TLRs) recognize microbial products, endogenous ligands (such as HGMB1, not shown) and synthetic agonists (not shown) either directly or aided by accessory molecules, such as CD14 and MD2. Dimerization of the receptor (heterodimerization for TLR2/6 and TLR1/6) is followed by downstream signaling. All TLRs engage the MyD88 adaptor molecule with the exception of TLR3, which signals through TRIF. TLR4 signaling can follow MyD88/TIRAP-dependent and/or TRIF/TRAM-dependent pathways. Activation of NF-κB, activator protein (AP)-1 and interferon regulatory factors (IRFs) induces expression of genes encoding inflammatory cytokines, type 1 IFN and IFN-inducible genes.
MyD88: Myeloid differentiation factor-88; TIRAP: TIR-associated protein; TRAM: Toll-receptor-associated molecule; TRIF: Toll receptor-associated-activator of interferon.

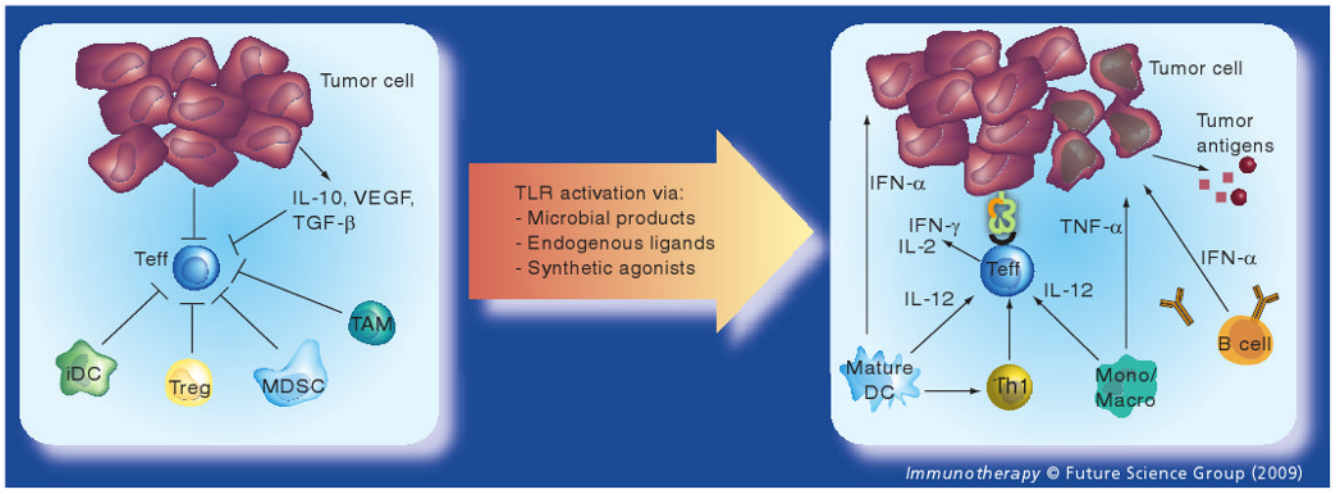

Figure 2. Toll-like receptor agonists and their immune effects on the tumor microenvironment

Left panel: several immunosuppressive mechanisms inhibit Teff function in the tumor microenvironment. Tumor-derived soluble factors such as IL-10, TGF-β and VEGF recruit MDSCs, prevent maturation of dendritic cells and inhibit Teff function. Furthermore, tumors deficient in B7–1 and B7–2 expression lead to inefficient T-cell stimulation; and expression of programmed death (PD)-ligand 1 on tumors inhibits T-cell activation (not shown). Tregs, often infiltrating tumors, can also directly suppress Teff function and trigger IL-10 production, which leads to upregulation of B7-H4 on antigen-presenting cells (APCs), and inhibition of DC maturation. B7-H4^{+} APCs such as TAM can induce cell cycle arrest in T cells. iDCs promote the accumulation of Tregs and result in T-cell anergy. Right panel: Toll-like receptor (TLR) activation enhances antitumor immunity via several mechanisms. TLR-mediated DC activation leads to enhanced phagocytosis, maturation with upregulation of MHC and costimulatory molecules (CD80, CD86 and CD40), secretion of Th1 cytokines (especially IL-12) and antigen presentation to lymphocytes resulting in the generation of Teff and antigen-specific B cells. Furthermore, local TLR activation can enhance natural killer cell-mediated cytotoxicity (not shown), upregulate MHC class I on tumor cells, directly induce tumor cell apoptosis and shift the tumor microenvironment toward an inflammatory milieu conducive to antigen cross-presentation and infiltration by functional Teff.

DC: Dendritic cell; iDC: Immature dendritic cell; MDSC: Myeloid-derived suppressor cell; Mono/Macro: Monocyte/macrophage; TAM: Tumor-associated macrophage; Teff: Effector T cell; Treg: Regulatory T cell.

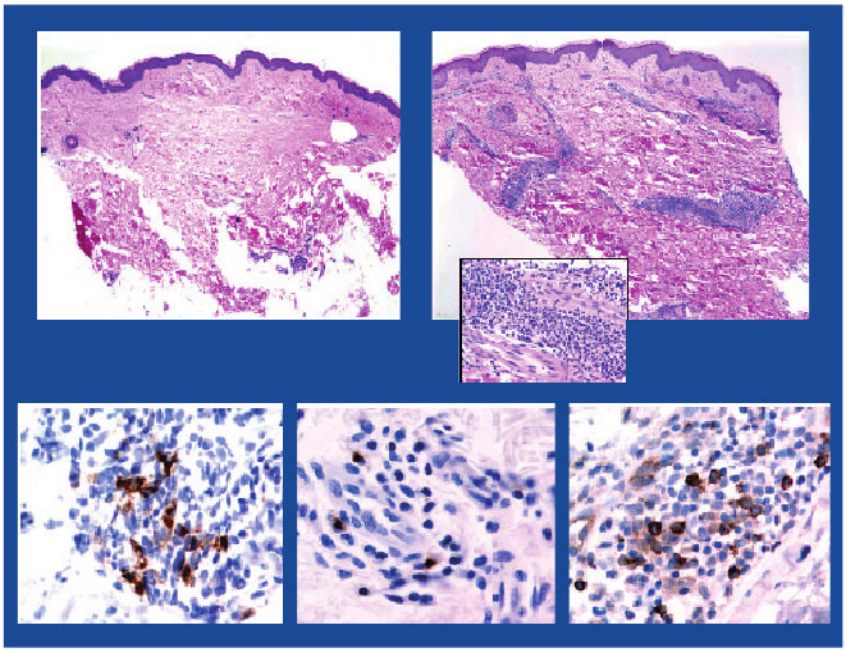

Figure 3. Dendritic cells recruited into skin treated with a Toll-like receptor-7 agonist

Imiquimod 5%, applied topically to patients' healthy skin for five days (in order to condition the site for vaccination) results in marked cellular infiltration in the dermis (H&E-stained sections, ×40 magnification). Upper left panel: untreated skin (control), Upper right panel: imiquimod-treated skin with perivascular and periadnexal mononuclear cell infiltrates (insert ×200 magnification). Infiltrates were mainly composed of lymphocytes as well as myeloid and plasmacytoid dendritic cells (as shown in representative immunohistochemistry sections). Lower left panel: mature DC (CD83), Lower center panel: activated myeloid dendritic cells (DC-LAMP) and Lower right panel: plasmacytoid dendritic cells (CD123).

Table 1

Toll-like receptor agonists under clinical investigation for cancer therapy

TLR	Agent	As monotherapy	As vaccine adjuvant	As *ex vivo* dendritic cell stimulator	In combination with chemotherapy	In combination with radiotherapy	In combination with targeted therapies, monoclonal antibodies or cytokines
2/4/9	BCG	Bladder cancer*, malignant melanoma [46]	Malignant melanoma [87], colon cancer [88], urothelial cancer [89], breast cancer [152]		Breast cancer [153], acute myelogenous leukemia [154]		
3	Poly I:C, Poly ICLC	Advanced cancers [80], malignant melanoma [82]	Malignant glioma (I/II), malignant melanoma (I/II), HCGβ-expressing tumors (I)	Malignant melanoma [112]			
4	MPL		NSCLC [96,98] and (III), malignant melanoma [90,91,99] and (III), breast and ovarian cancer [93,94], breast cancer (III), mucin-expressing tumors [92], ras-mutated tumors [95]				
4	LPS			Ductal carcinoma *in situ* [113], NHL (I/II), malignant melanoma (I/II)			
7	Imiquimod	Basal cell carcinoma*, actinic keratosis*, breast cancer (II), malignant melanoma (I/II)	Malignant melanoma [104]		Breast cancer (I/II)		Malignant melanoma [155]
7	852A	Advanced cancers [64,65]					
7/8	Resiquimod		Melanoma (I), HCGβ-expressing tumors (I)				
8	VTX-2337	Advanced cancers (I)					
9	CpG ODN	Glioblastoma [156], NHL [157], malignant melanoma [74,75], renal cell cancer (I), CTCL (I), CLL (I), malignant melanoma [75,77]	NY-ESO-1-expressing tumors [101], MAGE-3-expressing tumors (I/II), NSCLC (II/III), malignant melanoma [100], renal cell carcinoma (I/II)		NSCLC [133], colorectal cancer (I)	NHL (I/II)[120]	NSCLC (II), NHL [132,138, 158], NSCLC (I), colorectal cancer (I)

I/II/III indicates ongoing Phase I, II or III studies, as listed in [202].

*US FDA-approved for this indication.

852A: Synthetic imidazoquinoline mimicking viral ssRNA; VTX-2337: Small-molecule selective TLR8 agonist mimicking viral ssRNA; BCG: Bacillus of Calmette–Guerin, Mycobacterium bovis; CpG ODN: CpG oligodeoxynucleotide; CLL: Chronic lymphoid leukemia; CTCL: Cutaneous T-cell lymphoma; Imiquimod: Synthetic imidazoquinoline mimicking viral ssRNA; LPS: Lipopolysaccharide; MPL: Monophosphoryl lipid A; NHL: Non-Hodgkin's lymphoma; NSCLC: Non-small-cell lung cancer; Poly I:C: Polyriboinosinic-polyribocytidylic acid; PolyICLC: Poly I:C-poly-L-lysine; Resiquimod: Synthetic imidazoquinoline mimicking viral ssRNA; TLR: Toll-like receptor