Trial Watch: Immunostimulation with Toll-like receptor agonists in cancer therapy

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ABSTRACT

Accumulating preclinical evidence indicates that Toll-like receptor (TLR) agonists efficiently boost tumor-targeting immune responses (re)initiated by most, if not all, paradigms of anticancer immunotherapy. Moreover, TLR agonists have been successfully employed to ameliorate the efficacy of various chemotherapeutics and targeted anticancer agents, at least in rodent tumor models. So far, only three TLR agonists have been approved by regulatory agencies for use in cancer patients. Moreover, over the past decade, the interest of scientists and clinicians in these immunostimulatory agents has been fluctuating. Here, we summarize recent advances in the preclinical and clinical development of TLR agonists for cancer therapy.

Abbreviations: BCG, bacillus Calmette-Guérin; CRC, colorectal carcinoma; DAMP, damage-associated molecular pattern; DC, dendritic cell; FDA, Food and Drug Administration; HMGB1, high mobility group box 1; HPV, human papillomavirus; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MAMP, microbe-associated molecular pattern; MDC, myeloid-derived suppressor cell; MPL, monophosphoryl lipid A; NK, natural killer; NSCLC, non-small-cell lung carcinoma; ODN, oligodeoxynucleotide; OS, overall survival; PFS, progression-free survival; polyI:C, polyriboinosinic polyribocytidylic acid; TLR, Toll-like receptor

Introduction

The efficacy of various anticancer immunotherapies and chemotherapies relies on the (re)activation of robust tumor-targeting immune responses. Accumulating evidence indicates that artifically activating TLRs on myeloid components of the immune system, including monocytes, macrophages, and dendritic cells (DCs), constitutes an efficient strategy for boosting therapy-elicited anticancer immunity. Indeed, TLRs constitute a first-line of organismal defense against threats, and respond to danger signals (be them of microbial or endogenous origin) by promoting immune effector functions, including phagocytosis, antigen presentation, and cytokine secretion.

TLRs are highly conserved throughout evolution and can be found in species as distant as plants, flies, fish, and mammals. The human genome encodes 10 TLRs, each of which exhibits a peculiar pattern of expression and responds to specific signals. For illustrative purposes, TLRs can be subdivided into two major categories, depending on subcellular localization: (1) endosomal TLRs, including TLR3, TLR7, TLR8, TLR9, and TLR10 and (2) plasma-membrane-associated TLRs, including TLR1, TLR2, TLR4, TLR5, and TLR6. Most endosomal and plasma-membrane-associated human TLRs have been found to respond to conserved microbial products collectively known as microbe-associated molecular patterns (MAMPs), as well as to endogenous molecules collectively known as damage-associated molecular patterns (DAMPs). For instance, TLR4 can detect not only lipopolysaccharide (LPS), a component of the bacterial wall, but also high mobility group box 1 (HMGB1), a non-histone chromatin-binding protein that is released in the extracellular space upon cell death.

Preclinical and clinical evidence demonstrates that the release of TLR-activating DAMPs by dying cancer cells contributes to the elicitation of therapeutically relevant anticancer immune responses. In line with this notion, at least one TLR agonist that is currently approved by the US Food and Drug Administration (FDA) and equivalent agencies worldwide for use in humans mediates antineoplastic effects as standalone therapeutic intervention. This is the case of the so-called bacillus Calmette-Guérin (BCG), an attenuated variant of Mycobacterium bovis that is licensed for the treatment of non-invasive transitional cell carcinomas of the bladder.
note, three other TLR agonists are approved (at least in some countries) for use in oncological indications: (1) picibanil, a lyophilized preparation of Streptococcus pyogenes that is approved in Japan for the treatment of various carcinomas,\textsuperscript{2,26} (2) monophosphoryl lipid A (MPL), a derivative of Salmonella minnesota LPS that is employed as immunological adjuvant in a peptide-based vaccine specific for cervical carcinoma-associated strains of human papillomavirus (i.e., HPV-16 and HPV-18);\textsuperscript{27,28} and (3) imiquimod, an imidazoquinoline derivative that is used for the topical treatment of actinic keratosis, super-ficial basal cell carcinoma, and external genital/perianal warts (Condylomata acuminata).\textsuperscript{24,29} However, picibanil is invariably administered in association with chemotherapy, MPL aims at facilitating the induction of prophylactic antiviral immunity (which is associated with tumor prevention) rather than at boosting an immune response targeting formed cervical carcinomas, and the therapeutic activity of standalone Aldara\textsuperscript{6} (imiquimod 5% cream as commercialized by 3M Pharmaceuticals) may not depend on TLR7 signaling.\textsuperscript{30,31} Thus, BCG constitutes the only TLR agonist with unquestionable clinical activity as a standalone therapeutic intervention.

Here, we discuss recent progress on the development of TLR agonists for cancer therapy.

**Update on the development of TLR agonists for cancer therapy**

**Completed clinical studies**

Since the submission of our latest Trial Watch on this topic (May 2014),\textsuperscript{8} the results of six clinical trials investigating the therapeutic profile of experimental TLR agonists in cancer patients have been published in the peer-reviewed scientific literature (source http://www.ncbi.nlm.nih.gov/pubmed), and preliminary findings from two additional studies have been presented at the American Society of Clinical Oncology (ASCO) annual meeting (source http://meetinglibrary.asco.org/).

Smith and colleagues (Compass Oncology, Vancouver, WA, USA) tested increasing doses of the TLR9 agonist IMO-2055 (also known as EMD1201081),\textsuperscript{32-34} given in combination with erlotinib (a chemical receptor tyrosine kinase inhibitor)\textsuperscript{35} and bevacizumab (a monoclonal antibody specific for vascular endothelial growth factor A, VEGFA)\textsuperscript{36,37} to non-small-cell lung carcinoma (NSCLC) patients.\textsuperscript{38} Thirty-six individuals with NSCLC were enrolled in this Phase Ib study (NCT00633529), 35 of which received at least one treatment course. Two patients had to discontinue therapy owing to Grade 3 dehydration and fatigue, and four individuals experienced other serious adverse effects that did not require treatment discontinuation. Among 33 patients evaluable for response, 5 (15%) achieved partial response and 20 (61%) stable disease.\textsuperscript{38} These data indicate that IMO-2055 combined with erlotinib and bevacizumab is well tolerated by NSCLC patients and may exert clinical activity. The results of phase II–III studies are awaited to validate these findings.

Chan et al. (Vanderbilt-Ingram Cancer Center, Nashville, TN, USA) assessed the safety and efficacy of IMO-2055 combined with FOLFIRI (folic acid, 5-fluorouracil, irinotecan) and an FDA-approved epidermal growth factor receptor (EGFR)-targeting monoclonal antibody (cetuximab) in colorectal carcinoma (CRC) patients progressing after chemotherapy.\textsuperscript{39} Seventeen patients were enrolled in this open-label Phase Ib study (NCT00719199), sixteen of which received subcutaneous IMO-2055 weekly, at 0.16 mg/Kg, 0.32 mg/Kg, or 0.48 mg/Kg. One dose-limiting toxicity was observed (Grade 3 fatigue), at the lowest IMO-2055 dose. Additional common treatment-related adverse effects were mild injection site reactions, diarrhea, fatigue, hypomagnesemia, and stomatitis. One patient out of 16 achieved a partial response, and 12 had stable disease, 4 of which for more than 4 mo.\textsuperscript{39} These findings corroborate the safety and potential clinical activity of IMO-2055 in CRC patients co-treated with a FOLFIRI- and cetuximab-based immunotherapeutic regimen.

Arends and collaborators (Radboud University Medical Center, Nijmegen, The Netherlands) investigated the pharmacokinetics, pharmacodynamics, and activity of TMX-101 (a lipid preparation of imiquimod),\textsuperscript{40,41} administered intravesically as a standalone non-muscle-invasive bladder cancer.\textsuperscript{42} In this multicenter Phase I study, 23 individuals were initially assigned to receive 6 weekly instillations of 0.2% or 0.4% TMX-101, 21 of which completed treatment. Twenty patients (87.5%) experienced at least one adverse effect related to treatment, including urgency (34.8%), fatigue (21.7%), and bladder discomfort/pain (34.8%), but these were all mild (Grade 2 or lower). No clinical responses to treatment were achieved.\textsuperscript{42} The results of this study confirm the safety of intravesical TMX-101 for non-muscle-invasive bladder cancer patients.

Ursu et al. (Hôpital Avicenne, Bobigny, France) tested the safety profile of CpG-28 (a TLR9 agonist)\textsuperscript{43-45} administered subcutaneously and intrathecally to subjects with neoplastic meningitis.\textsuperscript{46} Twenty-nine patients were enrolled in this Phase I study and allocated to receive escalating doses of CpG-28 over 5 we following a Fibonacci design, alone or in combination with standard chemotherapy (at the physician’s discretion). Patients also received clobazam to prevent treatment-associated seizures and prednisolone to avoid arachnoiditis (i.e., a potentially lethal inflammation of the arachnoid). Only five Grade 3–4 toxicities were recorded, including confusion (in two patients), infection of the ventricular device (in two patients), and thrombocytopenia (in one patient). Median progression-free survival (PFS) and overall survival (OS) were 7 and 15 we, respectively, and the authors noted a tendency for improved PFS and OS in patients concomitantly treated with bevacizumab.\textsuperscript{46} These findings indicate that CpG-18 is well tolerated by patients with neoplastic meningitis, and call for the implementation of Phase II/III studies to better define the clinical profile of CpG-28 in cancer patients.

Schmoll and colleagues (University Clinic Halle, Halle, Germany) investigated the clinical profile of MGN1703 (a TLR9 agonist)\textsuperscript{47-49} in patients with metastatic CRC achieving disease control upon standard, first-line chemotherapy.\textsuperscript{50} Fifty-nine patients were enrolled in this Phase II, randomized, placebo-controlled study (NCT01208194), and were randomized 2:1 to receive 60 mg MGN1703 or placebo s.c., twice weekly until disease progression, withdrawal, or death. The most-frequent treatment-related adverse events were mild injection site reactions. Moreover, patients on maintenance therapy with
MGN1703 had an improved PFS as compared to patients receiving placebo (hazard ratio 0.49; 95% CI 0.26–0.94; \(p = 0.03\)), especially in the presence of activated natural killer T (NKT) cells. So far (median follow-up > 17 mo), no difference in OS between the study groups could be documented, but the trial has not yet been terminated.\(^5^0\) These findings indicate that maintenance MGN1703-based immunotherapy is well tolerated by CRC patients and sustain durable responses to first-line chemotherapy. Moreover, they suggest that the amount of activated NKT cells in the circulation may predict the response of CRC patients to MGN1703.

Weihrauch and collaborators (University of Cologne, Cologne, Germany) assessed the safety, pharmacokinetics, immunological effects, and preliminary clinical activity of escalating doses of MGN1703 in patients with metastatic solid tumors.\(^5^1\) Twenty-eight individuals affected by metastatic solid tumors were included in this open-label Phase I study (EudraCT 2007-006291-10), and they were assigned to receive 0.25, 2, 10, 30, or 60 mg MGN1703 s.c., either as a single dose or twice weekly for 6 we. Two severe drug-related adverse events were recorded: Grade 3 fatigue and prolonged activated partial thromboplastin time. The other toxicities were mild, encompassing injection-site reactions, dizziness, and nausea. Of 24 patients who could be evaluated for response, 6 achieved disease stabilization (3 of which for >12 we). Four patients receiving MGN1703 beyond the study protocol, as part of a compassionate use program, manifested stable disease for 18 mo. Of note, no immunological markers of response could be identified, perhaps with the exception of a non-significant increase in circulating TLR9-expressing B lymphocytes.\(^5^2\) These data indicate that administering 60 mg MGN1703 s.c. twice weekly is safe and has clinical activity in heavily pretreated individuals with metastatic solid neoplasms.

Bhatia et al. (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) are testing the clinical profile of G100 (a stabilized emulsion of the TLR4 agonist glycoppyranosyl lipid A),\(^5^3\) administered i.t. in a neoadjuvant setting to individuals with resectable Merkel cell carcinoma.\(^5^4\) To date, 8 out of 10 patients have been enrolled in this pilot study (NCT02035657), and all have completed one or more treatment cycles with G100. Only mild (Grade I-II) treatment-related adverse effects have been recorded for the moment, including injection site reactions and inflammation. Both patients with Stage IIIIB disease were recurrence-free 4 and 11 mo after treatment, and one of them experienced a pathological complete response after two courses of G100. Of six patients with Stage IV disease, two experienced disease stabilization after a single cycle of G100, and four had progressive disease.\(^5^5\) These results indicate that G100 has an acceptable safety and may exert clinical activity in Merkel cell carcinoma patients.

Bakhribah and colleagues (Roswell Park Cancer Institute, Buffalo, NY, USA) tested the safety, tolerability, pharmacokinetics, immunological activity, and preliminary antitumor potential of entolimod (a derivative of Salmonella flagellin also known as CBLB502),\(^5^6\) administered subcutaneously to subjects with various advanced malignancies, including NSCLC and CRC.\(^5^7\) Twenty-six patients were enrolled in this Phase I study (NCT01327236), and received entolimod s.c. over 2 we. Three dose-limiting toxicities were observed at the highest dose tested (40 μg/day), i.e., Grade 3 rigors and pyrexia, Grade 3 transaminitis and Grade 3 hypotension. Other common treatment-related adverse effects were fever, transient hypotension, and hyperglycemia. All patients exhibited signs of immune activation, and eight of them achieved disease stabilization.\(^6^2\) The authors of this study recommend to employ entolimod at a dose of 30 μg/day, and propose that entolimod can be safely employed alone or in combination with conventional chemotherapeutics, targeted anticancer agents or other forms of immunotherapy.

**Preclinical and translational advances**

A considerable amount of high-quality scientific reports dealing with TLR agonists and TLR signaling in preclinical and translational settings has been published in peer-reviewed journals during the last 15 mo (source http://www.ncbi.nlm.nih.gov/pubmed). Within such an abundant literature, we found of particular interest the works of: (1) Ohto and colleagues (University of Tokyo, Tokyo, Japan), who characterized three distinct structures of TLR9, unbound, bound to agonistic CpG oligodeoxynucleotides (ODNs), and bound to antagonistic DNA molecules;\(^6^3\) (2) Pickard and collaborators (University of Chicago, Chicago, IL, USA), who demonstrated that TLR signaling underlies a circuit that promotes host–commensal symbiosis during pathogen invasion, based on the release fucosylated proteins in the small intestine;\(^6^4\) (3) Bald and co-authors (University of Bonn, Bonn, Germany), who discovered that the release of HMGB1 from keratinocytes succumbing to UV irradiation stimulates a TLR4-dependent signal transduction cascade that favors the metastatic spreading of melanoma cells;\(^6^5\) (4) Liu et al. (University of Texas Southwestern Medical Center, Dallas, TX, USA), who found that TLR adaptor molecule 1 (TICAM1, also known as TRIF), the major transducer of TLR3-activating signals,\(^6^6\) activates downstream effectors including interferon regulatory factor 3 (IRF3) upon phosphorylation on conserved serine residues;\(^7^0\) (5) Scheeren and colleagues (Stanford University, Stanford, CA, USA), who elucidated a TLR2-dependent signaling pathway operating in mammary and intestinal epithelial cells to favor oncogenesis and tumor progression;\(^7^1\) (6) Dominguez-Villar and collaborators (Yale School of Medicine, New Haven, CT, USA), who implicated TLR7 in the anergic response of CD4\(^+\) T cells to chronic infection with RNA viruses (including HIV-1);\(^7^2\) (7) Skabytska and co-authors (Eberhard Karls University, Tübingen, Germany), who demonstrated that bacterial products recognized by TLR2-TLR6 heterodimers (but not TLR2-TLR1 heterodimers) exert immunosuppressive effects by favoring the expansion of myeloid-derived suppressor cells (MDSCs);\(^7^3\) (8) Kobayashi et al. (National Center for Global Health and Medicine, Tokyo, Japan), who identified the lysosomal protein solute carrier family 15 (oligopeptide transporter), member 4 (SLC15A4) as an obligatory factor for TLR7- and TLR9-driven Type I interferon (IFN) production in plasmacytoid DCs;\(^7^4\) (9) Nair-Gupta and colleagues (Icahn School of Medicine at Mount Sinai, New York, NY, USA), who found that TLR signaling is required for efficient cross-presentation as it drives the recruitment of MHC Class I molecules to phagosomes;\(^7^5\) (10) Rutkowski and collaborators (The Wistar Institute, Philadelphia, PA, USA), who reported that
commensal bacteria can drive malignant progression at extra-mucosal locations by favoring the interleukin-6 (IL-6)-dependent mobilization of MDSCs;76 (11) Yang and co-authors (The Feinstein Institute for Medical Research, Manhasset, NY, USA), who identified in lymphocyte antigen 96 (LY96, also known as MD-2) an essential component of the TLR4-dependent molecular machinery that senses the oxidized (pro-inflammatory) variant of HMGB1;77 (12) Nothelfer et al. (Institut Pasteur, Paris, France), who characterized a TLR2-dependent signaling pathway whereby Shigella spp. kill B lymphocytes;78 (13) Mancek-Keber and colleagues (National Institute of Chemistry, Ljubljana, Slovenia), who found that TLR4 can respond to exosomes bearing oxidized phospholipids, de facto implicating TLR4 in the detection of oxidative stress;79 (14) Daniele and collaborators (Georgetown University Medical Center, Washington, DC, USA), who identified oligomeric α-synuclein (a protein involved in Parkinson disease) as a novel agonist of TLR2-TLR1 heterodimers;80 (15) Kolb and co-authors (University of Louisville School of Medicine, Louisville, KY, USA), who discovered that Type I IFN can bias TLR4 toward using TRIF, as opposed to myeloid differentiation primary response 88 (MYD88), as a main signal transducer;81 and (16) Chatterjee et al. (Center de Recherche des Cordeliers, Paris, France), who found that TLR7 is expressed by NSCLC cells, contributing to tumor progression and resistance to therapy.82

These findings offer novel insights into several aspects of the TLR biology that are relevant for cancer therapy. In particular, they emphasize the complexity of the TLR system, which stems not only from the presence in the human genome of 10 distinct TLRs that can operate as homodimers or heterodimers (with different binding profiles), but also from the TLR expression pattern, which involves immune as well as non-immune cell populations, including cancer cells themselves. These two points should be taken under attentive consideration when novel TLR agonists are developed, and when preclinical and clinical data obtained with TLR ligands are interpreted.

**Recently initiated clinical trials**

Since the submission of our latest Trial Watch dealing with this topic (May 2014),8 no less than 48 clinical studies involving the administration of one or more TLR agonists to cancer patients have been initiated (source http://clinicaltrials.gov/). The majority of these trials involves the FDA-approved molecules BCG (9 studies) and imiquimod (14 studies), and the hitherto experimental TLR3 agonist HiltonolTM, a particular formulation of polyribosinic polyribocytidylic acid (polyI:C) that includes carboxymethylcellulose and poly-L-lysine as stabilizing agents83,85 (12 studies). BCG and imiquimod are often employed as on-label interventions, as (part of) the treatment given to the control arm of the study. Conversely, HiltonolTM is generally employed as an adjuvant, to improve the immunostimulatory activity of peptide-based anticancer vaccines,86 or DC-based interventions.87,88 In particular, BCG is being administered (1) as active comparator to assess the efficacy of hyperthermic mitomycin C-based chemotherapy in patients with urological tumors (NCT02254915); (2) as a standalone intravesical immunotherapeutic intervention to subjects with bladder carcinoma (NCT02281383; NCT02365207); (3) as a standalone subcutaneous immunotherapeutic agent to bladder carcinoma patients (NCT02326168); (4) intravesically, in combination with a highly active form of IL-15 (NCT02138734), mitomycin C-based chemotherapy (NCT02202044), a monoclonal antibody specific for programmed cell death 1 (PDCD1)89,90 (NCT02324582) or hyaluronic acid (NCT02207608) to subjects with bladder carcinoma; and (5) as a subcutaneous adjuvant for an intravenous vaccine against several pathogens to lung carcinoma patients (NCT02333474). In addition, a genetically modified variant of BCG (known as VPM1002BC) is being tested as standalone intravesical immunotherapeutic intervention in individuals with recurrent, non-muscle invasive bladder carcinoma (NCT02371447) (Table 1).

Topical imiquimod is being employed (1) as an active comparator in patients with actinic keratosis (NCT02404389), or genital warts (NCT02482428); (2) as a standalone immunotherapeutic intervention in subjects with cervical intraepithelial neoplasia (NCT02329171), lentigo maligna (NCT02394132), mycosis fungoides (NCT02301494), and extramammary Paget disease (NCT02385188); (3) after tumor excision or debulking in individuals with cervical intraepithelial neoplasia (NCT02329171) and nodular basal cell carcinoma (NCT02242929); (4) as an adjuvant to peptide-based vaccines, administered alone or together with additional chemo- or immunotherapeutic interventions, in patients with gastric or breast carcinomas (NCT02276300), glioma (NCT02454634), and prostate carcinoma (NCT02234921; NCT02293707; NCT02452307); (5) in combination with multimodal therapy in HIV-1+ individuals with anal high-grade squamous intraepithelial lesions (NCT02135419); and (6) in combination with photodynamic therapy in subjects with actinic keratoses (NCT02281682) (Table 1).

The safety and therapeutic potential of HiltonolTM is being assessed in (1) melanoma patients, who receive HiltonolTM in combination with a peptide-based vaccine (NCT02425306), DC-based intervention (NCT02334735) or a multimodal immunotherapeutic regimen encompassing a peptide-vaccine, ipilimumab (an FDA-approved monoclonal antibody that inhibits cytotoxic T lymphocyte-associated protein 4, CTLA4)91,92 and an experimental immunostimulatory monoclonal antibody targeting CD2793 (NCT02413827); (2) individuals with breast carcinoma (NCT02427581), glioma (NCT0258187), glioblastoma (NCT02149225; NCT02510950), CRC (NCT02134925), or NSCLC (NCT02495636), who are treated with HiltonolTM in combination with a peptide-based vaccine (optionally adjuvanted with recombinant granulocyte-macrophage colony-stimulating factor, GM-CSF); (3) subjects with gynecological tumors, receiving HiltonolTM in the context of a cancer cell lysate-based vaccination protocol94 (NCT02452775); (4) patients with neuroectodermal malignancies, who are treated with intramuscular HiltonolTM together with systemic decitabine-based chemotherapy (NCT02332889); and (5) individuals with advanced or metastatic solid tumors, who receive HiltonolTM as a standalone intramuscular immunotherapeutic intervention (NCT02423863) (Table 1).

The safety and efficacy of G100 are being assessed in (1) follicular lymphoma patients, who receive G100 as a standalone intratumoral immunotherapeutic intervention
Clinical trials recently started to investigate the therapeutic profile of Toll-like receptor agonists in cancer patients. Table 1. Clinical trials recently started to investigate the therapeutic profile of Toll-like receptor agonists in cancer patients.

| Molecule | Indication(s) | Phase | Status | Route | Notes | Ref. |
|----------|---------------|-------|--------|-------|-------|------|
| Ampligen™ | Ovarian carcinoma | I/II | Active, not recruiting | Intraperitoneal | In combination with DC-based vaccination | NCT02320305 |
| | Peritoneal tumors | I/II | Recruiting | Intravenous | In combination with DC-based vaccination | NCT02151448 |
| AS15 | Breast carcinoma | I | Recruiting | Intramuscular | Combined with peptide-based vaccination | NCT02364492 |
| BCG | Bladder carcinoma | I | Recruiting | Intravesical | Combined with lambramilumab | NCT02324582 |
| | | I/II | Recruiting | Intravesical | Combined with IL-15-based immunotherapy plus GM-CSF | NCT02138734 |
| | | II | Completed | Intravesical | Alone or combined with hyaluronic acid | NCT02207608 |
| | | II | Not yet recruiting | Subcutaneous | As standalone intervention | NCT02326168 |
| | | II | Recruiting | Intravesical | As standalone intervention | NCT02281383 |
| | | | Recruiting | Intravesical | In combination with mitomycin C-based chemotherapy | NCT02020444 |
| | Lung carcinoma | I/II | Recruiting | Subcutaneous | As standalone intervention | NCT02365207 |
| | | | | | Combined with a multiplepeptide-based vaccine | NCT02333474 |
| | | | | | As active comparator | NCT02254915 |
| | Urological tumors | I/II | Recruiting | Intravesical | Combined with a multiplepeptide-based vaccine | NCT02452697 |
| DUK-CPG-001 | Hematological neoplasms | II | Not yet recruiting | Intravenous | In combination with NK cell-enriched DLI | NCT02365207 |
| | G100 | Follicular lymphoma | I/II | Recruiting | Intratumoral | As standalone intervention | NCT02501473 |
| | Melanoma | n.a. | Recruiting | Intramuscular | Combined with peptide-based vaccination | NCT02320305 |
| | Soft tissue sarcoma | I | Recruiting | Intramuscular | In combination with radiation therapy | NCT02180698 |
| | Solid tumors | I | Recruiting | Intramuscular | Combined with peptide-based vaccination | NCT02387125 |
| Hiltonol™ | Breast carcinoma | I | Not yet recruiting | n.a. | Combined with peptide-based vaccination plus GM-CSF | NCT02427581 |
| | Colorectal carcinoma | II | Recruiting | Subcutaneous | Combined with peptide-based vaccination | NCT02134925 |
| | Gynecological tumors | I | Recruiting | Subcutaneous | Combined with cancer cell lysate-based vaccination | NCT02452775 |
| | Glioblastoma | 0 | Not yet recruiting | n.a. | Combined with peptide-based vaccination plus GM-CSF | NCT02510950 |
| | | I | Recruiting | Subcutaneous | Combined with peptide-based vaccination plus GM-CSF | NCT02149225 |
| | Glioma | II | Recruiting | Intramuscular | Combined with peptide-based vaccination | NCT02358187 |
| | Melanoma | I/II | Recruiting | n.a. | Combined with peptide-based vaccination | NCT02425306 |
| | | II | Not yet recruiting | Subcutaneous | Combined with a DC-based vaccine | NCT02334735 |
| | | II | Recruiting | n.a. | Combined with ipilimumab, varililumab, and a peptide vaccine targeted to DCs | NCT02413827 |
| | Neuroectodermal tumors | II | Recruiting | Intramuscular | Combined with decitabine-based chemotherapy | NCT02332889 |
| | NSCLC | II | Active, not recruiting | Subcutaneous | Combined with peptide-based vaccination | NCT02495366 |
| | Solid tumors | I/II | Recruiting | Intramuscular | As standalone intervention | NCT02423863 |
| Imiquimod | Actinic keratosis | I | Recruiting | Topical | As active comparator | NCT02404389 |
| | | IV | Recruiting | Topical | Combined with PDT | NCT02281682 |
| | Anal intraepithelial lesions | III | Recruiting | Topical | Combined with multimodal therapy | NCT02135419 |
| | Breast carcinoma | Gastric carcinoma | I | Recruiting | Topical | Combined with peptide-based vaccination plus GM-CSF | NCT02276300 |
| | | Cervical intraepithelial lesions | III | Recruiting | Topical | Alone or upon resection | NCT02329171 |
| | Genital warts | II | Recruiting | Topical | As active comparator | NCT02481428 |
| | Glioma | I | Recruiting | Topical | Combined with peptide-based vaccination | NCT02454634 |
| | Lentigo maligna | III | Not yet recruiting | Topical | As standalone intervention | NCT02394132 |
| | Mycosis fungoides | n.a. | Not yet recruiting | Topical | As standalone intervention | NCT02301494 |
| | Nodular basal cell carcinoma | III | Not yet recruiting | Topical | After tumor debulking | NCT02242929 |
| | Paget disease | III | Recruiting | Topical | As standalone intervention | NCT02385188 |
| | Prostate carcinoma | I | Recruiting | Topical | Combined with peptide-based vaccination and chemotherapy | NCT02234921 |
| | | I/II | Active, not recruiting | Topical | Combined with peptide-based vaccination | NCT02452307 |
| | | | | | Combined with peptide-based vaccination | NCT02293707 |
| Motolimod | Ovarian carcinoma | I/II | Active, not recruiting | Subcutaneous | Combined with MEDI4736 and liposomal doxorubicin | NCT02431559 |
| SD-101 | Lymphoma | I/II | Recruiting | Intratumoral | Combined with ipilimumab and radiotherapy | NCT02254772 |
| | Melanoma | I | Active, not recruiting | Intratumoral | In combination with radiation therapy | NCT02266147 |
| | | | | | In combination with pembrolizumab | NCT02521870 |
| VPM1002BC | Bladder carcinoma | I/II | Recruiting | Intravesical | As standalone intervention | NCT02371447 |

Abbreviations: BCG, bacillus Calmette-Guérin; DC, dendritic cell; DLI, donor lymphocyte infusion; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; n.a., not available; NK, natural killer; NSCLC, non-small-cell lung carcinoma; PDT, photodynamic therapy.

* Initiated between 2014, May 1st and the day of submission.
combination with ipilimumab and/or radiation therapy in lymphoma patients (NCT02254772; NCT02266147); and (2) in combination with pembrolizumab (an FDA-approved anti-PDCD1 monoclonal antibody)\textsuperscript{90} in subjects with metastatic melanoma (NCT02521870). The TLR3 agonist Ampligen\textsuperscript{TM} (polyI:C)\textsuperscript{97,98} is being evaluated in (1) ovarian cancer patients, who are treated intraperitoneal Ampligen\textsuperscript{TM} in combination with a DC-based vaccine (NCT02432378); and (2) subjects with peritoneal malignancies, also receiving Ampligen\textsuperscript{TM} in support of DC-based vaccination (NCT02151448). Motolimod (a TLR8 agonist previously known as VTX-2337)\textsuperscript{99,100} is being tested in combination with liposomal doxorubicin (an inducer of immunogenic cell death)\textsuperscript{108,110-112} and MEDI4736 (a monoclonal antibody targeting CD274, the main PD-1 ligand)\textsuperscript{104-106} in patients with ovarian carcinoma (NCT02431559). The clinical profile of the novel TLR9 agonist DUK-CPG-001 is being investigated in subjects with hematological malignancies, who receive DUK-CPG-001 in support of natural killer (NK) cell-enriched lymphocyte donor infusions (NCT02452697). Finally, the multicomponent adjuvant AS15, which contains MPL, QS-21 (a water-soluble saponin extracted from the South American tree Quillaja saponaria Molina) and agatolimod (a CpG ODN), de facto operating as a mixed TLR2/TLR4/TLR9 agonist, is being tested in support of peptide-based vaccination in breast carcinoma patients (NCT02364492) (Table 1).

Of note, all these studies are open, with two notable exceptions: NCT02254915, which has been withdrawn owing to concerns on the availability of BCG on the market; and NCT02207608, which has been completed (source http://clinicaltrials.gov/). NCT02207608 investigated the influence of hyaluronic acid on the local toxicities of BCG-based immunotherapy in bladder carcinoma patients. Thirty subjects were enrolled in this pilot study and randomized 1:1 to receive intravesical BCG alone or combined with hyaluronic acid.\textsuperscript{107} BCG therapy had a lower impact on quality of life in patients concomitantly receiving hyaluronic acid, but similar therapeutic effects.\textsuperscript{107} These data suggest a possible role for hyaluronic acid in limiting the local side effects of BCG-based immunotherapy.

As for the studies discussed in our previous Trial Wheels dealing with TLR agonists,\textsuperscript{2,8,108,109} the following trials have changed status during the last 15 mo: NCT01303172, NCT01599818, NCT01266603, NCT01435356, NCT01834248, NCT02056557, NCT01748747, NCT00799110, NCT00821964, NCT00824733, NCT00986609, NCT01008527, NCT01149343, NCT01204684, NCT01204684, NCT01355393, NCT01437605, NCT01527136, NCT01666444, and NCT01677962, which are listed as “Active, not recruiting”; NCT00626483, NCT00778164, NCT01532960, NCT01543464, NCT01734564, NCT01808820, NCT01836029, NCT01861535, and NCT02077868, which are currently recruiting participants; NCT01118313, NCT00453050, NCT00671554, NCT00821652, NCT00948961, NCT01040832, NCT01079741, NCT01208194, NCT01245673, NCT01294293, NCT01334177, NCT01498172, NCT01539824, NCT01676831, and NCT01731652, which have been completed; NCT01745354, NCT01400672, and NCT01720836, which have been suspended; NCT01035216, NCT01289210, NCT01403285, NCT01743807, and NCT01984892, which have been terminated; and NCT01663558, which has been withdrawn (source http://clinicaltrials.gov/).

NCT01400672 (a Phase I trial investigating the therapeutic profile of a tumor lysate-based vaccine adjuvanted with imiquimod in pontine glioma patients) has been suspended because of an ongoing amendment in study protocol; NCT01720836 (a Phase I/II study assessing the clinical profile of a peptide-based vaccine adjuvanted with Hiltonol\textsuperscript{TM} in NSCLC patients) for undisclosed reasons; and NCT01745354 (a Phase I trial testing SD-101 in recurrent lymphoma patients after hematopoietic stem cell transplantation) because the principal investigator is currently on maternity leave. NCT00671554 (a Phase I/II trial testing intratumoral BCG injections combined with an autologous DC-based vaccine in Stage IV melanoma patients) has been terminated owing to business considerations; NCT01403285 (a Phase I study investigating the clinical profile of a multipeptide vaccine adjuvanted with imiquimod in glioblastoma patients) and NCT01289210 (a Phase I/II trial assessing the safety and efficacy of motolimod plus radiation therapy in patients with low-grade B-cell lymphoma) because of limited patient accrual; NCT01035216 and NCT01743807 (two Phase I studies testing a novel TLR9 agonist in leukemia patients) because the company producing the drug (GKKG168) has decided to no longer support the study; and NCT01984892 (a Phase II trial testing Hiltonol\textsuperscript{TM} as standalone intratumoral immunotherapeutic intervention in patients with solid tumors) at the discretion of the principal investigator. NCT01663558 (a Phase IV study comparing ablative therapy with imiquimod-based immunotherapy in HIV-1\textsuperscript{+} patients with anal dysplasia) has been withdrawn prior to enrollment as funds became unavailable (source http://clinicaltrials.gov/). The results of NCT00948961 (a Phase I/II trial assessing the therapeutic profile of a peptide-based vaccine adjuvanted with a TLR7 agonist and Hiltonol\textsuperscript{TM} in patients with advanced tumors), NCT01040832 (a Phase II study testing a TLR9 agonist in combination with a tumor-targeting monoclonal antibody in subjects with head and neck cancer), and NCT01245673 (a Phase 2 trial investigating a multimodal immunotherapeutic approach involving Hiltonol\textsuperscript{TM} in myeloma patients) have been published in peer-reviewed scientific journals.\textsuperscript{110-112} Conversely, to the best of our knowledge, the final results of NCT00118313, NCT00453050, NCT00821652, NCT01079741, NCT01208194, NCT01294293, NCT01334177, NCT01498172, NCT01539824, NCT01676831, and NCT01731652 have not been disseminated yet.

**Concluding remarks**

During the last 15 mo (May 2014–August 2015), approximately 50 clinical trials have been initiated to assess the safety and efficacy of immunotherapeutic interventions involving TLR agonists in cancer patients (Table 1). This marks a clear reversal in the number of recently initiated clinical studies involving TLR ligands. Recently, cancer immunotherapy in general has attracted renovate interest from experimental oncologists and
clinicians worldwide, especially after the approval of several checkpoint blockers (including ipilimumab and pembrolizumab) for the treatment of melanoma patients. However, whether the clinical success of checkpoint blockers underlies the rediscovery of other forms of immunotherapy remains unclear. Irrespective of this unknown, the results of recently initiated clinical trials involving TLR agonists are urgently awaited. These findings may confirm that the use of TLR agonists in cancer therapy truly stands at a dead end, as it seemed until a few months ago, or may pave the way to the development of novel immunotherapeutic antineoplastic regimens in which TLR agonists play a protagonist role.

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No potential conflict of interest were disclosed.

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