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REVIEW

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; MDSC, 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

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Introduction

The efficacy of various anticancer immunotherapies and chemotherapies relies on the (re)activation of robust tumor-targeting immune responses.[1-4] Accumulating evidence indicates that artificially 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.[5-8] 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.[9,10]

TLRs are highly conserved throughout evolution and can be found in species as distant as plants, flies, fish, and mammals.[11-13] 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.[9,10,14,15] 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,[16] but also high mobility group box 1 (HMGB1), a non-histone chromatin-binding protein that is released in the extracellular space upon cell death.[17-20]

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.[21-23] 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.[24,25] Of

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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;[5,26] (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);[27,28] and (3) imiquimod, an imidazoquinoline derivative that is used for the topical treatment of actinic keratosis, superficial basal cell carcinoma, and external genital/perianal warts (*Condylomata acuminata*).[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® (imiquimod 5% cream as commercialized by 3M Pharmaceuticals) may not depend on TLR7 signaling.[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),[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),[32-34] given in combination with erlotinib (a chemical receptor tyrosine kinase inhibitor)[35] and bevacizumab (a monoclonal antibody specific for vascular endothelial growth factor A, VEGFA)[36,37] to non-small-cell lung carcinoma (NSCLC) patients.[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.[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 (folinic 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.[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 patients out of 16 achieved a partial response, and 12 had stable disease, 4 of which for more than 4 mo.[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 liquid preparation of imiquimod),[40,41] administered intravesically as a standalone immunotherapeutic intervention to subjects with papillary non-muscle-invasive bladder cancer.[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.[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)[43-45] administered subcutaneously and intrathecally to subjects with neoplastic meningitis.[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 thromboneutropenia (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.[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)[47-49] in patients with metastatic CRC achieving disease control upon standard, first-line chemotherapy.[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.[50] 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.[51] 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.[51] 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 glucopyranosyl lipid A),[52-55] administered *i.t.* in a neoadjuvant setting to individuals with resectable Merkel cell carcinoma.[56] 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 IIIB 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.[56] 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),[57-61] administered subcutaneously to subjects with various advanced malignancies, including NSCLC and CRC.[62] Twenty-six patients were enrolled in this Phase I study (NCT01527236), 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.[62] 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;[63] (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;[64] (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;[65] (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,[66-69] activates downstream effectors including interferon regulatory factor 3 (IRF3) upon phosphorylation on conserved serine residues;[70] (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;[71] (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);[72] (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);[73] (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;[74] (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;[75] (10) Rutkowski and collaborators (The Wistar Institute, Philadelphia, PA, USA), who reported that

commensal bacteria can drive malignant progression at extramucosal 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 Hiltonol^{TM}, a particular formulation of polyriboinosinic polyribocytidylic acid (polyI:C) that includes carboxymethylcellulose and poly-*L*-lysine as stabilizing agents[83-85] (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, Hiltonol^{TM} 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 (NCT022 07608) 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 (NCT0 2329171) 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 keratosis (NCT02281682) (Table 1).

The safety and therapeutic potential of Hiltonol^{TM} is being assessed in (1) melanoma patients, who receive Hiltonol^{TM} 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 CD27[93] (NCT02413827); (2) individuals with breast carcinoma (NCT02427581), glioma (NCT0 2358187), glioblastoma (NCT02149225; NCT02510950), CRC (NCT02134925), or NSCLC (NCT02495636), who are treated with Hiltonol^{TM} in combination with a peptide-based vaccine (optionally adjuvanted with recombinant granulocyte-macrophage colony-stimulating factor, GM-CSF); (3) subjects with gynecological tumors, receiving Hiltonol^{TM} in the context of a cancer cell lysate-based vaccination protocol[94] (NCT024 52775); (4) patients with neuroectodermal malignancies, who are treated with intramuscular Hiltonol^{TM} together with systemic decitabine-based chemotherapy (NCT02332889); and (5) individuals with advanced or metastatic solid tumors, who receive Hiltonol^{TM} 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

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	NCT02432378
	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	In combination with lambrolizumab	NCT02324582
		I/II	Recruiting	Intravesical	Combined with IL-15-based immunotherapy	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
		n.a.	Recruiting	Intravesical	In combination with mitomycin C-based chemotherapy	NCT02202044
					As standalone intervention	NCT02365207
	Lung carcinoma	I/II	Recruiting	Subcutaneous	Combined with a multipeptide-based vaccine	NCT02333474
	Urological tumors	III	Withdrawn	Intravesical	As active comparator	NCT02254915
DUK-CPG-001	Hematological neoplasms	II	Not yet recruiting	Intravenous	In combination with NK cell-enriched DLIs	NCT02452697
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	Intratumoral	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	NCT02427581
	Colorectal carcinoma	II	Recruiting	Subcutaneous	Combined with peptide-based vaccination	NCT02134925
	Gynecological tumors	I	Recruiting	n.a.	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	In combination with a DC-based vaccine	NCT02334735
		II	Recruiting	n.a.	Combined with ipilimumab, varlilumab, 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	NCT02495636
	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	NCT02482428
	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
		II	Recruiting	Topical	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
					In combination with radiation therapy	NCT02266147
	Melanoma	I	Active, not recruiting	Intratumoral	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.

(NCT02501473); (2) individuals with soft tissue sarcoma, who are treated with intratumoral G100 combined with radiation therapy (NCT02180698); and (3) subjects with melanoma (NCT02320305) or advanced solid tumors (NCT02387125), in whom intramuscular G100 is employed to adjuvant a peptide-based anticancer vaccine. Intratumoral SD-101 (a phosphorothiolate CpG ODN that operates as a TLR9 agonist)[95,96] is being tested (1) in

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)[90] in subjects with metastatic melanoma (NCT02521870). The TLR3 agonist Ampligen™ (polyI:C)[97,98] is being evaluated in (1) ovarian cancer patients, who are treated intraperitoneal Ampligen™ in combination with a DC-based vaccine (NCT02432378); and (2) subjects with peritoneal malignancies, also receiving Ampligen™ in support of DC-based vaccination (NCT02151448). Motolimod (a TLR8 agonist previously known as VTX-2337)[99,100] is being tested in combination with liposomal doxorubicin (an inducer of immunogenic cell death)[18,22,101-103] and MEDI4736 (a monoclonal antibody targeting CD274, the main PD-1 ligand)[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 toxicites 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.[107] BCG therapy had a lower impact on quality of life in patients concomitantly receiving hyaluronic acid, but similar therapeutic effects.[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 Watches dealing with TLR agonists,[7,8,108,109] the following trials have changed status during the last 15 mo: NCT01303172, NCT01559818, NCT01266603, NCT01435356, NCT01834248, NCT02035657, NCT01748747, NCT00799110, NCT00821964, NCT00824733, NCT00986609, NCT01008527, NCT01149343, NCT01204684, NCT01204684, NCT01355393, NCT01437605, NCT01527136, NCT01666444, and NCT01677962, which are listed as "Active, not recruiting"; NCT00626483, NCT00788164, NCT01532960, NCT01543464, NCT01734564, NCT01808820, NCT01836029, NCT01861535, and NCT02077868, which are currently recruiting participants; NCT00118313, 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™ 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 (GNKG168) has decided to no longer support the study; and NCT01984892 (a Phase II trial testing Hiltonol™ 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^+ 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™ 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™ in myeloma patients) have been published in peer-reviewed scientific journals.[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 trend we monitored throughout the previous 24 mo (May 2012–May 2014), which were characterized by a steady decrease in the number of recently initiated clinical studies involving TLR ligands. Recently, cancer immunotherapy in general has attracted renovated 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. [113-117] 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.

Disclosure of potential conflicts of interest

No potential conflict of interest were disclosed.

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