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Trial Watch

Experimental Toll-like receptor agonists for cancer therapy

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Abbreviations: CTL, cytotoxic lymphocyte; DAMPs, damage-associated molecular patterns; DC, dendritic cell; dSLIM, DNA-based double stem-loop immunomodulator; dsRNA, double-stranded RNA; EGFR, epidermal growth factor receptor; GM-CSF, granulocyte monocyte colony-stimulating factor; HNSCC, head and neck squamous cell carcinoma; IFN, interferon; IL, interleukin; IMO, immunomodulatory oligonucleotide; ISS, immunostimulatory sequence; LBP, LPS-binding protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAL, MYD88 adapter-like; MALP-2, macrophage-activating lipopeptide 2; MAMPs, microbe-associated molecular patterns; MPL, monophosphoryl lipid A; NSCLC, non-small cell lung carcinoma; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cell; polyA:U, polyadenylic polyuridylic acid; polyI:C, polyriboinosinic polyribocytidylic acid; PRR, pattern recognition receptor; SIV, simian immunodeficiency virus; STAT1, signal transducer and activator of transcription 1; TIL, Toll/Interleukin 1 receptor-like; TNFα, tumor necrosis factor α; TLR, Toll-like receptor, Treg, FOXP3$^+$ immunosuppressive T cell

Toll-like receptors (TLRs) are prototypic pattern recognition receptors (PRRs) best known for their ability to activate the innate immune system in response to conserved microbial components such as lipopolysaccharide and double-stranded RNA. Accumulating evidence indicates that the function of TLRs is not restricted to the elicitation of innate immune responses against invading pathogens. TLRs have indeed been shown to participate in tissue repair and injury-induced regeneration as well as in adaptive immune responses against cancer. In particular, TLR4 signaling appears to be required for the efficient processing and cross-presentation of cell-associated tumor antigens by dendritic cells, which de facto underlie optimal therapeutic responses to some anticancer drugs. Thus, TLRs constitute prominent therapeutic targets for the activation/intensification of anticancer immune responses. In line with this notion, long-used preparations such as the Coley toxin (a mixture of killed *Streptococcus pyogenes* and *Serratia marcescens* bacteria) and the bacillus Calmette-Guérin (BCG, an attenuated strain of *Mycobacterium bovis* originally developed as a vaccine against tuberculosis), both of which have been associated with consistent anticancer responses, potently activate TLR2 and TLR4 signaling. Today, besides BCG, only one TLR agonist is FDA-approved for therapeutic use in cancer patients: imiquimod. In this Trial Watch, we will briefly present the role of TLRs in innate and cognate immunity and discuss the progress of clinical studies evaluating the safety and efficacy of experimental TLR agonists as immunostimulatory agents for oncological indications.

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Introduction

The *Toll* gene was first identified in 1985 as a controller of the dorsal-ventral embryonic polarity in the fruit fly *Drosophila melanogaster*.[1,2] A few years later, *Toll* was shown to encode a transmembrane protein with a large extracellular domain containing at least 15 repeats of a 24 residue-long leucine-rich sequence.[3] However, it was not until 1996 that Jules Hoffmann's laboratory characterized the critical role of Toll in the response of *D. melanogaster* to fungal infections.[4] In 1991, Gay and Keith discovered that the intracellular tails of Drosophila Toll and of the human receptor for interleukin-1 (IL-1) share consistent homology,[5] yet the first human ortholog of Toll (initially named TIL for Toll/Interleukin 1 receptor-like) was discovered as late as in 1994,[6] and mapped to chromosome 4p14 in 1996.[7] In 1997, another member of the Toll-like receptor (TLR) family, namely TLR4, was shown to control the expression of genes involved in innate immunity.[8] One year later, two independent laboratories demonstrated that TLR2 and TLR4 mediate the activation of innate immunity in response to bacterial lipopolysaccharide (LPS).[9,10] Since then, 13 different TLRs have been characterized in mammals (10 in humans), and members of the TLR family have been identified in organisms as evolutionary distant from mammals as plants and pufferfish.[11-13] The discovery of TLRs has granted to Jules Hoffmann and Bruce Beutler the 2011 Nobel prize in Medicine or Physiology.

TLRs are single membrane-spanning proteins that are devoid of enzymatic activity but can bind so-called pathogen-associated molecular patterns (PAMPs), i.e., conserved microbial components including bacterial (e.g., LPS and other constituents of the

cell wall) and viral (e.g., double-stranded RNA, dsRNA) products.[14] As TLRs recognize microbial products irrespective of their pathogenic potential, it has recently been suggested to abandon the term PAMP in favor of the more correct variant MAMP, standing for "microbe-associated molecular pattern."[15] According to currently accepted models, TLRs operate as homo- or hetero-dimers and are expressed either at the plasma membrane (TLRs that mainly bind proteo-lipidic MAMPs, i.e., TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) or in endosomes (TLRs that detect microbial nucleic acids, i.e., TLR3, TLR7, TLR8, TLR9).[16] TLR10, which is the only orphan receptor among human TLRs, has also been shown to co-localize with TLR2 at phagosomes,[17-19] suggesting that it may share with TLR2 the ability to bind acylated lipopeptides.[20] Conclusive data on this issue, however, have not yet been reported.

TLR11–13 are not encoded in the human genome. In mice, TLR11–13 are constitutively expressed in the central nervous system and undergo several-fold induction in response to cysticercosis.[21] TLR11 reportedly recognizes a profilin-like protein expressed by *Toxoplasma gondii* and has been localized at the endoplasmic reticulum.[22-24] TLR13 also appears to be localized intracellularly, where it would specifically detect the vesicular stomatitis virus.[25] So far, the ligand specificity and intracellular localization of TLR12 remain unexplored.

Ligand-bound TLR dimers can recruit the adaptor proteins MYD88 and TIRAP (also known as MYD88 adaptor-like, MAL), in turn driving the assembly of a multiprotein signaling complex that—among other effects—activates the transcriptional factor NFκB (which regulates a large number of pro-inflammatory cytokines).[26,27] MYD88 is employed as an obligate or facultative adaptor by all human TLRs but TLR3. TLR3 dimers signal indeed via a MYD88-independent molecular cascade, involving the protein TICAM1 (also known as TRIF) and resulting in the activation of the transcription factor IRF7 (which regulates the expression of Type I interferons, IFNs, and other IFN-inducible genes).[28-30] Such a MYD88-independent signaling pathway can also be engaged by TLR4, as demonstrated by the fact that TICAM1-deficient mice exhibit defects in both TLR3- and TLR4-mediated IRF activation.[29] Of note, mice lacking TICAM2 (a TICAM1-related protein also known as TRAM), exhibit defective cytokine production in response to TLR4 (but not TLR3) ligands, indicating that TICAM1 is required—but not sufficient—to transduce MYD88-independent signals elicited by TLR4 (but not by TLR3).[31] Recent data demonstrate that MYD88 and TIRAP actively inhibit TLR3 signaling,[32,33] corroborating the notion that TLR3 constitutes a rather peculiar member of the TLR family, sharing limited functional properties with its homologs. Of note, most TLRs are predominantly expressed in immune cells (e.g., monocytes, mature macrophages, mast cells and dendritic cells, DCs) and/or in cells involved in the first-line defense against infection (e.g., intestinal epithelial cells) (**Table 1**).

Importantly, TLRs (in particular TLR2 and TLR4) bind not only to microbial products but also to a wide array of endogenous damage-associated molecular patterns (DAMPs), i.e., molecules that are released or exposed by stressed or dying cells and act as endogenous danger signals to promote sterile inflammation.[34] These products encompass heat-shock proteins (e.g., HSP60, HSP70),[35,36] the non-histone chromatin-binding protein HMGB1,[37,38] uric acid,[39] the surfactant protein A,[40] as well as several components or breakdown products of the extracellular matrix.[41] Specific DAMPs, including (though perhaps not limited to) the endoplasmic reticulum protein calreticulin (CRT),[42,43] ATP,[44-46] HMGB1[37] and HSP70,[47] are generated—in a highly defined spatiotemporal pattern—by cancer cells as they succumb to radiotherapy or to a restricted group of chemotherapeutic agents (i.e., mitoxantrone, cyclophosphamide, oxaliplatin and doxorubicin).[48,49] By binding to pattern-recognition receptors (PRRs) expressed on the surface of cells from the immune system (notably DCs), such a spatiotemporally defined combination of signals stimulates tumor-specific immune responses that, at least in some settings, underlie the success of anticancer therapy.[49,50] In this context, TLR4 appears to bind both HSP70 and HMGB1, thus enhancing the processing of tumor antigens by DCs and their cross-presentation to T cells.[37,47,51] In line with these observations, loss-of function polymorphisms of *TLR4* have been shown to negatively influence the response of breast cancer patients to adjuvant radiotherapy or anthracyclin-based chemotherapy.[37] Thus, TLRs are crucial not only for the orchestration of innate responses to infectious agents but also for the optimal activation of the immune system against cancer.

Given these premises, it was no surprise to discover that long-used remedies such as the Coley toxin (a mixture of killed *Streptococcus pyogenes* and *Serratia marcescens* bacteria) and the bacillus Calmette-Guérin (BCG, an attenuated strain of *Mycobacterium bovis* initially developed as an anti-tuberculosis vaccine), both of which have been associated with consistent anticancer responses, are potent activators of TLR2 and TLR4[52,53] that operate in a MYD88-dependent fashion.[54] Along similar lines, imiquimod (a small imidazoquinoline derivative originally developed as a topic antiviral agent) was found to activate the TLR7-MYD88 axis, leading to the production of IFNα, tumor necrosis factor α (TNFα) and IL-6, as late as in 2002, five years after its initial approval by FDA for the treatment of actinic keratosis (a precancerous lesion of the skin).[55] The use of the Coley toxin has been discontinued in the early 1960s, due to concerns following the thalidomide case (thalidomide was withdrawn 11 y after its approval as it was found to be highly teratogenic, leading to more than 10,000 children born with deformities worldwide).[56] Conversely, both BCG and imiquimod are currently approved by FDA, the former for the immunotherapy of in situ bladder carcinoma and the latter against actinic keratosis, superficial basal cell carcinoma, and external genital warts (condylomata acuminata).[57] Importantly, monophosphoryl lipid A (MPL), a derivative of the LPS of *Salmonella minnesota* that functions as a potent agonist or the TLR4-TICAM1 signaling axis,[58] is approved by FDA for use in humans as part of Cervarix®, a vaccine against human papillomavirus 16 and 18 (the etiological agents of about 70% of cervical carcinoma cases).[59]

In this Trial Watch, we will review the results of recently completed clinical trials and discuss the progress of ongoing

Table 1. Expression, localization and ligand specificity of human TLRs

TLR	MAMP	DAMP	Localization	Expression	Ref.
TLR1	Lipoproteins Triacylated lipopeptides Zymosan	Unknown	Plasma membrane	B cells Mature macrophages Monocytes	260,261
TLR2	Acylated lipopeptides Amphotericin B Atypical LPS Byglican Forins Glyco(phospho)lipids Lipoteichoic acid Peptidoglycan Phenol soluble modulin Zymosan	Heat-shock proteins Surfactant protein A Uric acid	Plasma membrane	DCs Mast cells Mature macrophages Monocytes	34-36, 39-41, 60-62, 262
TLR3	Unknown	dsRNA	Endosome	B cells DCs Epithelial cells Endothelial cells	16,91-93
TLR4	Glycolipids LPS	Biglycan Fibrinogen Fibronectin Heparan sulfate HMGB1 Heat-shock proteins Hyaluronic acid Surfactant protein A Uric acid	Plasma membrane	DCs IECs Mast cells Mature macrophages Monocytes	10,16, 35-38, 40,47,64
TLR5	Flagellin	Unknown	Plasma membrane	DCs IECs Mature macrophages Monocytes	16,235-237
TLR6	Diacylated lipopeptides Lipoproteins Lipoteichoic acid Zymosan	Unknown	Plasma membrane	B cells Mast cells Mature macrophages Monocytes Neutrophils	61,62, 263,264
TLR7	ssRNA of microbial origin	Unknown	Endosome	B cells Mature macrophages Monocytes Plasmacytoid DCs	16,154,155
TLR8	ssRNA of microbial origin	Unknown	Endosome	Mast cells Mature macrophages Monocytes	16,154,155
TLR9	Unmethylated CpG-rich DNA	Unknown	Endosome	B cells Mature macrophages Monocytes Plasmacytoid DCs	16,178, 179,265
TLR10	Acylated lipopeptides	Unknown	Plasma membrane	B cells Plasmacytoid DCs	17-20

Abbreviations: DAMP, damage-associated molecular pattern; DC, dendritic cell; dsRNA, double-stranded RNA; HMGB1, high mobility group B1; IEC, intestinal epithelial cell; LPS, lipopolysaccharide; MAMP, microbe-associated molecular pattern; ODN, oligodeoxynucleotide; ssRNA, single-stranded RNA.

studies that have employed/are employing experimental TLR agonists as immunostimulatory agents for oncological indications.

TLR2 and TLR4 Agonists

TLR2 and TLR4 are predominantly expressed on the surface of monocytes, mature macrophages and DCs.[16] Assisted by CD36, homodimeric TLR2 specifically recognizes products from Gram-positive bacteria, including lipoteichoic acid (LTA).[60] As an alternative, TLR2 can heterodimerize with TLR1 or TLR6, thereby acquiring the capacity to bind triacylated lipopeptides, such as the synthetic molecule Pam_3CSK_4, and biacylated lipopeptides, like the natural occurring macrophage-activating lipopeptide 2 (MALP-2), respectively.[61,62] Contrarily to TLR2,

TLR4 binds components from Gram-negative bacteria, mainly LPS, with the assistance of CD14.[63] This process is highly facilitated by the LPS-binding protein (LBP), an acute phase soluble protein that binds LPS and favors its interaction with the TLR4/CD14 complex.[64]

SMP-105 is a TLR2-specific agonist consisting of cell wall skeleton components (mainly mycolic acid and peptidoglycans) isolated from *M. bovis* (strain BCG Tokyo).[65] Peritoneal exudate cells from *Tlr2*$^{-/-}$ and *Myd88*$^{-/-}$ (but not *Tlr4*$^{-/-}$) mice fail to secrete TNFα and IL-6 in response to SMP-105, confirming the strict TLR selectivity of this agent.[66] Mice receiving inactivated Lewis lung carcinoma cells together with SMP-105 develop an intense immune response featuring high levels of IFNγ-producing tumor-specific cytotoxic lymphocytes (CTLs).[66] Such a response has been shown to efficiently control the growth of implanted tumors.[66] Thus, SMP-105 exerts potent immunostimulatory effects in vivo. Nevertheless, the development of SMP-105 has not yet reached the clinic.

The anticancer properties of LPS, the natural ligand of TLR2 and TLR4 also known as endotoxin, have been discovered as early as in the 1960s,[67,68] when the existence of TLRs was not even suspected. Since then, dozens of studies have confirmed that the administration of LPS, either alone or in combination with other anticancer agents, efficiently induce tumor regression in murine models.[69-71] Early clinical trials sought to determine the safety of LPS, with particular attention to the fact that circulating LPS might cause the sequential release into the circulation of large amounts of pro-inflammatory cytokines, a potentially lethal process that is known as endotoxic shock.[72,73] These studies determined that low doses of LPS are relatively safe, in particular if preceded by a bolus of the non-steroidal anti-inflammatory drug ibuprofene, even though virtually all patients receiving LPS showed increased circulating levels of pro-inflammatory cytokines including TNFα and IL-1.[74,75] A few years later, the combination ibuprofen + LPS (purified from *Salmonella abortus equi*) was tested in colorectal cancer patients, showing encouraging results, as well as in non-small cell lung carcinoma (NSCLC) patients, for whom the therapeutic benefit was marginal.[76] Today, official sources list only three clinical trials investigating the efficacy of LPS in oncological indications: one as part of a complex cell-based vaccination approach to treat Ewing sarcoma, rhabdomyosarcoma and neuroblastoma patients (NCT00923351), one in which LPS is co-administered with a peptide vaccine and a mineral oil-based adjuvant to melanoma patients (NCT01585350), and one in which LPS is combined with peptide-pulsed DCs for the treatment of hematological malignancies (NCT00923910) (source www.clinicaltrials.gov).

Picibanil (OK-432) is a lyophilized preparation of group A Streptococcus (namely *Streptococcus pyogenes*) that has been approved for use as an anticancer adjuvant as early as in 1975, yet only by the Japanese Ministry of Health and Welfare.[41,77] Picibanil was soon found to induce potent Th1 immune responses in vivo, in mice, but its ability to bind and activate TLR4 on the surface of DCs has been unveiled only a few years ago.[78,79] Since its approval by local authorities, picibanil has been extensively used in Japan for the treatment of cervical, gastric and oral squamous cell carcinoma.[80-82] In the US and Europe, picibanil remains an investigational drug, and has recently been tested for its ability to boost immune responses in cancer patients treated with whole protein-based vaccines (NCT00291473). The results of this study, however, have not yet been divulgated. In addition, picibanil is currently being investigated in combination with a preoperative intratumoral injection of DCs in patients with resectable pancreatic cancer (NCT00795977), and associated to DCs and chemotherapy (cyclophosphamide + docetaxel) in head and neck squamous cell carcinoma (HNSCC) patients (NCT01149902) (source www.clinicaltrials.gov).

OM-174 (also known as CRX-527) is a water soluble, diphosphorylated and triacetylated form of lipid-A purified from *Escherichia coli*.[83] OM-174, which functions as a mixed TLR2/TLR4 agonist,[84] has been shown to exacerbate the efficacy of cyclophosphamide against murine melanoma B16 cells growing in immunocompetent mice,[85] and to exert radiosensitizing effects in vivo, in an IFNγ-dependent fashion.[86] Relatively recent publications indicate that the safety and efficacy of OM-174 have been tested in cancer patients.[16,87] However, we were unable to find additional sources that would report the results of these trials. Now, the clinical development of OM-174 has been discontinued (source www.clinicaltrials.gov).

In summary, while many pure TLR4 and mixed TLR2/TLR4 agonists (including BCG, MPL and picibanil) have already been approved for use in humans by governmental agencies, the clinical development of pure TLR2 agonists as adjuvants to boost anticancer immune responses now appears to stand at an impasse. At least in part, this may be due to the fact that TLR2 agonists promote tumor infiltration by FOXP3^{+} T cells (Tregs) and stimulate the production of IL-10, de facto exerting local and systemic immunosuppressive effects.[88,89] Along similar lines, the possibility to employ TLR4 antagonists for cancer-unrelated indications (mainly sepsis) raised initial enthusiasms that often have been deceived.[16] One notable exception is provided by the small chemical ibudilast (AV411),[90] which is currently being tested in (a few) Phase I/II clinical trials for the treatment of addiction withdrawal and headache (source www.clinicaltrials.gov).

Table 2 summarizes recent clinical progress of experimental TLR2/TLR4 agonists for oncological indications.

TLR3 Agonists

TLR3 is an endosomal TLR primarily expressed by B cells, myeloid DCs as well as by epithelial and endothelial cells.[16] At an acidic pH (such as that found in the endosomal lumen), TLR3 binds 40–50 nucleotide-long dsRNA molecules, interacting almost exclusively with the sugar phosphate backbone rather than with bases.[91-93] Intriguingly, the interaction of dsRNA with TLR3 does not trigger conformational changes, but only facilitate/stabilize homodimerization, de facto bringing the intracellular tails of TLR3 monomers in close proximity of each other.[93] Of note, in some epithelial cell types including breast cancer cells, ligand-bound TLR3 can stimulate the intrinsic pathway of apoptosis[94-96] or trigger a chemokine cascade that orchestrates the recruitment of CTLs to the tumor bed.[97,98]

Table 2. Clinical trials evaluating TLR2/TLR4 agonists in oncological indications*

Compound	Indication	Trials	Phase	Status	Notes	Ref.
LPS	Ewing's sarcoma Neuroblastoma Rhabdomyosarcoma	1	I-II	Suspended	Combined with 8H9 mAb, IL-4 and KLH-pulsed DCs vaccine	NCT00923351
	Hematological malignancies	1	I-II	Recruiting	Combined with IL-4, KLH and a WT1-pulsed DC-based vaccine	NCT00923910
	Melanoma	1	I	Not yet recruiting	Combined with a multipeptide vaccine	NCT01585350
Picibanil	Pancreatic cancer Head and neck carcinoma	1 1	I-II I	Active, not recruiting Recruiting	Combined with preoperative intratumoral injections of DCs Combined with chemo-immunotherapy	NCT00795977 NCT01149902

Abbreviations: DC, dendritic cell; IL-4, interleukin 4; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; LPS, lipopolysaccharide. *Started after January, 1st 2008.

Ampligen (also known as rintatolimod) consists in polyriboinosinic polyribocytidylic acid (polyI:C), a synthetic dsRNA molecule originally developed in the late 1960s to exacerbate antiviral responses.[99] In vitro, Ampligen not only optimally activates DCs (be them derived from healthy donors or cancer patients) to promote cytotoxic Th1 responses,[100] but also exerts direct cytostatic/cytotoxic effects against a wide spectrum of tumor cells.[101,102] In vivo, Ampligen has been shown to suppress the growth of a large panel of neoplasms, in both (at least partially) immunodeficient[103-106] and immunocompetent models.[107-110] In the latter scenario, Ampligen appears to operate in a multimodal fashion, encompassing the activation of natural killer (NK) cells[107] the conversion of tumor-promoting macrophages into their tumor-suppressing counterparts,[110] as well as direct cytostatic/cytotoxic effects on cancer cells.[111] Of note, all these responses have been shown to be abolished in *Ticam1*$^{-/-}$, but not in *Myd88*$^{-/-}$, mice.[107,110] Recently, Shir et al. have demonstrated that an epidermal growth factor receptor (EGFR)-targeted variant of Ampligen is highly efficient in activating (implanted) human immune cells to destroy pre-established disseminated EGFR-overexpressing tumors, with no adverse toxic effects.[112] This suggests that specifically targeting TLR3 ligands to tumor cells might increase their therapeutic potential. Early clinical trials in oncological settings demonstrate that Ampligen is well tolerated, even over long administration periods (> 1 y), and that patients normally do not develop neutralizing antibodies.[113] Today, Ampligen is being tested in combination with a peptide vaccine in HER2-positive breast cancer patients (NCT01355393), with autologous tumor cell lysates in patients affected by reproductive tract neoplasms (NCT01312389), or with celecoxib, IFNα and Hiltonol (see below) in resectable colorectal cancer patients (NCT01545141) (source www.clinicaltrials.gov).

Hiltonol (also known as poly-ICLC) is a particular formulation of polyI:C that includes carboxymethylcellulose and poly-L-lysine as stabilizing agents.[114] Hiltonol appears to be 5 to 10 times more resistant to degradation by serum hydrolases than Ampligen, and exhibits a denaturation temperature about 40°C higher.[114] In line with this notion, Hiltonol has been shown to significantly elevate the levels of circulating IFNγ in non-human primates in conditions in which an equivalent dose of Ampligen failed to do so,[114] and to exert potent immunostimulatory effects in multiple preclinical models, including mice, rats, rabbits, monkeys and chimpanzees.[114-118] Such encouraging preclinical results generated a consistent wave of Phase I/II clinical trials that assessed the safety and efficacy of Hiltonol (often as a standalone agent or combined with vaccination protocols) in multiple, very heterogeneous oncological indications, encompassing hematological malignancies,[119-122] brain tumors,[120,123-128] melanoma,[129] ovarian carcinoma,[130] renal cancer[121,131] and other advanced neoplasms.[132-135] With the exception of a single study, reporting substantial toxicity and no clinical benefits in ovarian carcinoma patients,[130] these trials concluded that Hiltonol is safe and moderately efficient in boosting anticancer immune responses. More recently, Hiltonol has been investigated as a single agent or combined to radiotherapy in B- and T-cell lymphoma patients (NCT00880867), in combination with a chimeric vaccine and resiquimod (see below) in patients affected by bladder carcinoma (NCT01094496),[136] as well as together with a peptide vaccine in patients bearing neoplasms of the reproductive tract. The former two studies have been terminated for unreported reasons, whereas the latter has been completed, though the results have not yet been divulgated. Nowadays, the safety and efficacy of Hiltonol are being investigated in around 20 Phase I/II clinical trials. In all but two cases, one in which Hiltonol is used as a single agent in glioma patients (NCT1188096) and one in which it is combined with celecoxib, IFNα and Ampligen (see above) in resectable colorectal cancer patients (NCT01545141), Hiltonol is employed as an adjuvant to peptide cell-based anticancer vaccines (source www.clinicaltrials.gov). Major oncological indications in which Hiltonol is being tested include brain malignancies (6 trials), melanoma (4 trials), breast carcinoma (2 trials) and colorectal cancer (2 trials).

The high molecular mass dsRNA mimic IPH-3102 has been shown to promote NFκB activation and to sustain Type I IFN responses in vitro, hence exerting cytotoxic effects against melanoma and breast cancer cells. In addition, IPH-3102 mediates consistent immunostimulatory effects in vivo, in mice.[16,137] Still, IPH-3102 has not yet entered (and presumably will never enter) clinical trials.

Polyadenylic polyuridylic acid (polyA:U) is a synthetic dsRNA molecule that has been shown to exert immunostimulatory effects in vivo as early as in 1967.[138] Still, polyA:U has not been

recognized as a TLR3 agonist until 2008, when polyA:U was reported to stimulate myeloid DCs to produce IL-12.[139] When combined with anticancer vaccines, poly(A:U) promotes Th1 responses that control tumor growth and are associated with the establishment of immunological memory.[140] However, TLR3 is expressed not only in immune cells but also in epithelial, endothelial and cancer cells,[96,141-143] Thus, poly(A:U) can exert, at least in some settings, pro-tumor functions,[144] raising the need for compensatory strategies that would uncouple the immunostimulatory (and therapeutically beneficial) effects of TLR3 agonists from their pro-tumorigenic potential.[97] During the last three decades, poly(A:U) has been demonstrated to be safe but variably efficient for the adjuvant therapy of resectable breast cancer (as a single agent or in combination with radiotherapy),[145,146] locally advanced gastric cancer (combined with 5-fluorouracil and doxorubicin),[147,148] resectable colorectal carcinoma (as a standalone agent),[149] and other not better specified solid malignancies.[150,151] Of note, the therapeutic benefits of poly(A:U) in combination with locoregional radiotherapy as an adjuvant treatment of breast cancer patients, as reported by Laplanche et al.,[146] have recently been shown to be restricted to patients bearing TLR3$^+$ tumors.[98] This indicates that TLR3 expression levels might constitute a predictive biomarker for the efficacy of poly(A:U), at least in some oncological setting. Today, the development of poly (A:U) appears to have come at an impasse, as there are no clinical trials evaluating its efficacy for oncological as well as for cancer-unrelated indications (source www.clinicaltrials.gov).

Table 3 summarizes recent clinical progress of experimental TLR3 agonists for oncological indications.

TLR7 and TLR8 Agonists

TLR7 and TLR8 are predominantly expressed at the endosomal membrane of monocytes and macrophages, plasmacytoid DCs (constituting one peculiar subset of DCs that operate at the interface between innate and adaptive immunity),[152] and mast cells.[16] Moreover, both these endosomal TLRs are highly expressed by bronchial epithelial cells (especially TLR7) and NSCLC cells (especially TLR8), in situ.[153] TLR7 and TLR8 bind single-stranded RNA molecules of viral origin, the species specificity relying on the elevated content of uridine and guanosine nucleosides that characterize viral—as compared with mammalian—RNAs.[154,155] Binding is inhibited by specific chemical modifications of the RNA molecule (such as the 2'-O-methylation of uridine and guanosine nucleosides).[156] This is critical as it allows RNA interference to be used in vivo in the absence of massive DC activation and inflammation.[156] Of note, TLR7 stimulation has been associated with the development of tolerance, which can be circumvented by the use of appropriate treatment schedules.[157]

Resiquimod (R-848) is a small imidazoquinoline that exerts potent immunostimulatory effects, both in vitro[158-160] and in vivo,[161,162] by operating as a TLR7/TLR8 agonist.[55] Thus, similar to its FDA-approved structural analog imiquimod, topical resiquimod efficiently enhances immune responses to local antigens, leading to a consistent increase in the frequency of antigen-specific CTLs.[163] Resiquimod has initially been investigated for the therapy of herpes simplex virus, yielding promising results,[164] and hepatitis C virus, in this case being associated with dose-limiting toxicity similar to that of IFNα.[165] The development of resiquimod as an anti-herpetic agent has been suspended due to lack of efficacy in Phase III clinical trials.[16] However, following encouraging results obtained in patients affected by actinic keratosis,[166] and perhaps with an influence from the clinical success of imiquimod, resiquimod has recently been tested on patients affected by common warts (which are caused by human papillomaviruses). The results of these six studies, all of which are listed as completed by official sources, have not yet been published. In addition, resiquimod has been evaluated in combination with a chimeric vaccine and Hiltonol (see above) in patients affected by bladder carcinoma (NCT01094496),[136] a study that has been terminated for unreported reasons. Today, resiquimod is under investigation as a single agent for the treatment of actinic keratosis (NCT01583816), cutaneous T-cell lymphoma (NCT01497795) and melanoma (NCT00470379), as well as in combination with multiple vaccination strategies in melanoma patients (NCT00960752) and individuals bearing not better specified NY-ESO-1-expressing tumors (NCT00948961, NCT00821652) (source www.clinicaltrials.gov).

852A is another member of the imidazoquinoline group that functions as a TLR7 agonist.[167] 852A potently stimulates plasmacytoid DCs to produce IFNα as well as several other immunostimulatory cytokines, in vitro and in vivo, thus driving Th1 immune responses that—at least in some models—can exert antitumor effects.[167-169] Early clinical trials that investigated the safety and pharmacokinetic profile of 852A reported no major side effects,[170] and pointed to subcutaneous delivery as an acceptable means for obtaining systemic responses.[171] More recently, intravenous delivery has been preferred as a method to systemically activate the immune system in cancer patients,[172] even though prolonged schedules have been associated with a high incidence of mild-to-severe side effects (NCT00319748).[173] In addition, the anticancer potential of 852A (always employed as a standalone therapy) has been investigated in unresectable melanoma patients (NCT00091689, NCT00189332), in individuals affected by Barrett's esophagus with high-grade dysplasia (NCT00386594), in patients with hematological malignancies (NCT00276159), as well as in subjects affected by not better specified refractory solid tumors (NCT00276159). Though official sources list these studies as completed, their results have not yet been disclosed (source www.clinicaltrials.gov).

VTX-2337 is a small molecule that specifically binds and activates TLR8.[174] The preclinical characterization of VTX-2337 appears to be scarce. Indeed, we found a single scientific work reporting that VTX-2337 stimulates the production TNFα and IL-12 from monocytes and myeloid DCs, as well as the secretion of IFNγ by NK cells, in vitro.[174] Nevertheless, VTX-2337 has already entered Phase I/II clinical trials for oncological indications. In particular, the safety and dose-dependent pharmacological profile of VTX-2337 have been demonstrated in a cohort of patients affected by advanced solid tumors (NCT00688415) (ref. 175 and Annual ASCO Meeting 2011, Abstract #2537). A Phase I/II clinical

Table 3. Clinical trials evaluating TLR3 agonists in oncological indications*

Compound	Indication	Trials	Phase	Status	Notes	Ref.
Ampligen	Breast cancer	1	I-II	Recruiting	Combined with GM-CSF and a HER-2-based peptide vaccine	NCT01355393
	Colorectal cancer	1	I-II	Not yet recruiting	Combined with celecoxib, Hiltonol and IFNα2b	NCT01545141
	Reproductive tract cancer	1	I-II	Active, not recruiting	Combined with tumor cell lysates and multiple adjuvants	NCT01312389
Hiltonol	Advanced cancer	1	I-II	Recruiting	Combined with CDX-1401 ± resiquimod	NCT00948961
	B- and T-cell lymphoma	1	I	Terminated	As single agent or combined with local radiotherapy	NCT00880867
	Bladder cancer	1	II	Terminated	Combined with CDX-1307 vaccine, chemotherapy, GM-CSF and resiquimod	NCT01094496
	Brain tumors	6	n.a.	Recruiting		NCT01130077
			0	Active, not recruiting	Combined with a HLA-A2-restricted glioma antigen-based vaccine	NCT00795457
			0	Active, not recruiting		NCT00874861
			I-II	Active, not recruiting	Combined with DC-based vaccine	NCT00766753
			II	Recruiting	As single agent	NCT01188096
			II	Recruiting	Combined with autologous tumor cell lysate-pulsed DCs	NCT01204684
	Breast cancer	2	n.a.	Not yet recruiting	Combined with a multipeptide vaccine	NCT01532960
			0	Recruiting	Combined with MUC-1-based vaccine	NCT00986609
	Colorectal cancer	2	I-II	Not yet recruiting	Combined with celecoxib, Ampligen and IFNα2b	NCT01545141
			II	Recruiting	Combined with MUC-1-based vaccine	NCT00773097
	Melanoma	4	I	Not yet recruiting	Combined with a multipeptide vaccine	NCT01585350
			I	Recruiting	Combined with a CD40 agonist and various peptides	NCT01008527
			I-II	Recruiting	Combined with a NY-ESO-1-based vaccine ± oil adjuvant	NCT01079741
			II	Recruiting	Combined with a multipeptide vaccine	NCT01437605
	Myeloma	1	II	Recruiting	Combined with autologous SCT, lenalidomide and a multipeptide vaccine	NCT01245673
	Pancreatic cancer	1	I	Recruiting	Combined with peptide-pulsed DCs	NCT01410968
	Prostate cancer	1	n.a.	Recruiting	Combined with a multipeptide vaccine	NCT00694551
	Reproductive tract cancer	1	I-II	Completed	Combined with NY-ESO-1 overlapping peptide and oil adjuvant	NCT00616941

Abbreviations: DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; HER2, human epidermal growth factor receptor 2; IFN, interferon; n.a., not available; SCT, stem cell transplantation. *Started after January, 1st 2008.

trial in which VTX-2337 was supposed to be used in combination with radiotherapy for the treatment of low-grade B-cell lymphoma has been recently terminated, due to insufficient rates of recruitment (NCT01289210). A similar trial (NCT01396018) is still listed as active by official sources, though the last update is nearly one year old. Thus, VTX-2337 is currently being evaluated in two Phase I/II studies only: in combination with the anti-EGFR antibody cetuximab for the treatment of advanced, recurrent or metastatic HNSCC (NCT01334177) and associated to doxorubicin in patients affected by tumors of the reproductive tract or peritoneal cavity (NCT01294293).

The clinical development of the TLR7 agonists AZD8848 (DSP-3025) and ANA773, as well as that of the TLR8 agonist VTX-1463, which until recently were being tested for the treatment of allergic asthma, allergic rhinitis and hepatitis C virus infection,[16,176,177] has been discontinued (source www.clinicaltrials.gov).

TLR7 and TLR8 agonists have recently been shown to increase the resistance of cultured NSCLC cells to chemotherapy, an effect that ensued the activation of a MYD88-NFκB signaling axis resulting in the upregulation of anti-apoptotic BCL-2 proteins.[153] Of note, NSCLC cells treated in vitro with TLR7 and TLR8

agonists exhibit a gene expression signature that is very similar to that of untreated NSCLC cells in situ, suggesting that the latter might be chronically stimulated by (endogenous?) TLR ligands.[153] These observations suggest that the use of TLR7/TLR8 agonists as adjuvants in anticancer therapy might be beneficial to patients affected by specific neoplasms but detrimental to others, notably to patients whose tumors express high amounts of TLR7 and/or TLR8.

Table 4 summarizes recent clinical progress of experimental TLR7/TLR8 agonists for oncological indications.

TLR9 Agonists

TLR9 is mainly found in the endosomal compartment of B cells, monocytes, macrophages and plasmacytoid DCs.[16] The main ligand of TLR9 is bacterial/viral DNA, differing from its mammalian counterpart for the elevated frequency of unmethylated CpG dinucleotides.[178] Indeed, whereas mammalian DNA has no immunostimulatory activity, the administration of bacterial/viral DNA induces a potent Th1 immune response in vivo, which is entirely abrogated in *Tlr9*$^{-/-}$ mice.[178] Of note, a TLR9 variant that is ectopically expressed at the plasma membrane has been shown to recognize not only bacterial/viral but also mammalian DNA,[179] indicating that the basis for the discrimination between self and exogenous DNA mainly resides in the subcellular localization of TLR9 rather than in biochemical features (i.e., the methylation state and frequency of CpG islands) of its ligand.

CpG-28, CpG-685 (GNKG168) and CpG-7909 (PF-3512676, Agatolimod, Promune®) are class B unmethylated CpG oligonucleotides, i.e., CpG oligonucleotides that—due to precise structural features such a length comprised between 18 and 28 nucleotides, a fully phosphorothioated backbone and the presence of one or more 5'-RYCGYR-3' 6-mers (R = A or G; Y = T or C)—strongly stimulate B cells and monocytes.[180] In some cell types including malignant B cells, TLR9 stimulation by type B CpG oligonucleotides induces significant morphologic, phenotypic and functional alterations that are followed by a signal transducer and activator of transcription 1 (STAT1)-dependent program of apoptotic cell death.[181] In line with this notion, human leukemic cells that are pretreated in vitro with type B CpG oligonucleotides lose the ability to generate overt leukemia upon inoculation into immunodeficient mice.[181] Moreover, the administration of type B CpG oligonucleotides (alone or combined with chemotherapeutics or peptide vaccines) to tumor-bearing rodents reportedly exerts potent anticancer effects.[181-185] Initial Phase I/II clinical trials to test the safety and efficacy of CpG-7909 for oncological indications were launched in April 2000.[186] Approximately in the same period, CpG-7909 begun to be extensively investigated as an adjuvant for cancer-unrelated indications (mainly antiviral vaccines), showing no severe side effects and encouraging efficacy.[187-189] In humans, the subcutaneous (more than the intravenous) administration of CpG-7909 activates both myeloid and plasmacytoid DCs,[190] resulting in a potent systemic Th1 response that can boost anticancer immunity.[191-193] During the last decade, the safety and anticancer potential of CpG-7909 (as a standalone agent or in combination with chemotherapy and/or vaccination approaches) have been investigated in a large number of Phase I/II clinical trials, including studies with leukemia,[194] lymphoma,[195-198] basal cell carcinoma,[199] melanoma,[199-202] esophageal squamous cell carcinoma,[203] NSCLC,[204-206] renal cell carcinoma,[207] and prostate cancer patients.[208] In a peculiar approach, CpG-7909 has been tested as an adjuvant for oncological indications in combination with MPL

Table 4. Clinical trials evaluating TLR7/TLR8 agonists in oncological indications*

Compound	Indication	Trials	Phase	Status	Notes	Ref.
TLR7/ TLR8 agonists						
Resiquimod	Actinic keratosis	1	II	Not yet recruiting	As single agent	NCT01583816
	Advanced cancer	1	I-II	Recruiting	Combined with CDX-1401 ± Hiltonol	NCT00948961
	Bladder cancer	1	II	Terminated	Combined with CDX-1307 vaccine, chemotherapy, GM-CSF and Hiltonol	NCT01094496
	CTCL	1	I-II	Recruiting	As single agent	NCT01497795
	Melanoma	1	II	Recruiting	Combined with a gp100- and MAGE-3-based vaccine	NCT00960752
	Various neoplasms	1	I	Unknown	Combined with a NY-ESO-1-based vaccine	NCT00821652
TLR8 agonists						
VTX-2337	Advanced cancer	1	I	Completed	As single agent	NCT00688415
	B-cell lymphoma	2	I-II	Recruiting Terminated	Combined with local radiotherapy	NCT01396018 NCT01289210
	HNC	1	I	Recruiting	Combined with cetuximab	NCT01334177
	Reproductive tract tumors	1	I	Recruiting	Combined with doxorubicin	NCT01294293

Abbreviations: CTCL, cutaneous T-cell lymphoma; HNC, head and neck carcinoma; GM-CSF, granulocyte macrophage colony-stimulating factor. *Started after January, 1st 2008.

and QS-21, a water soluble saponin extracted from the South American tree *Quillaja saponaria* Molina.[209] Such a formulation, known as AS15 or GSK2302025A, has been evaluated for its capacity to boost immune responses in melanoma patients receiving recombinant MAGE-A3 (see refs. 210, 211 and Annual ASCO Meeting 2008, Abstracts 9045 and 9065). In the vast majority of cases, CpG-7909 and AS15 were found to be safe and to exert moderate anticancer effects, de facto supporting subsequent clinical trials based on larger patient cohorts. One of these Phase III studies has already been terminated, demonstrating that the addition of CpG-7909 to gemcitabine + cisplatin in NSCLC patients improves neither progression-free nor overall survival.[212] Following the disclosure of these negative results, the evaluation of CpG-7909 in NSCLC patients has been discontinued. Nevertheless, CpG-7909 remains under investigation in a few Phase I/II clinical trials, evaluating it in combination with radiotherapy in B-cell lymphoma patients (NCT00880581), combined with the anti-HER2 antibody trastuzumab in breast cancer patients (NCT00824733), and as an adjuvant to anticancer vaccination protocols in patients bearing esophageal malignancies (NCT00669292), or not better specified NY-ESO-1-expressing tumors (NCT00819806). Along similar lines, AS15 is now being extensively tested in multiple myeloma (NCT01380145), melanoma (NCT01149343, NCT01266603, NCT01425749, NCT01437605) and bladder cancer (NCT01435356, NCT01498172) patients, always in combination with MAGE-A3-based vaccines, as well as in metastatic breast cancer patients (NCT00952692), combined with a peptide vaccine targeting HER2. At odds with the large number of studies performed on CpG-7909 and AS15, the safety and efficacy of CpG-685 (as a single agent) are currently being investigated in a single Phase I clinical trial, enrolling patients with relapsed/refractory B-cell chronic lymphocytic leukemia (NCT01035216). Although the results of Phase I/II clinical trials testing CpG-28 in recurrent glioblastoma patients seemed encouraging,[213,214] today official sources list no active studies that would evaluate this compound in oncological settings (source www.clinicaltrials.gov).

By virtue of synthetic CpR dinucleotides, the immunomodulatory oligonucleotide 2055 (IMO-2055, also known as EMD1201081) mimics unmethylated CpG sequences and hence functions as a potent TLR9 agonist.[215,216] In vivo, IMO-2055 induces Th1 immune responses that are associated with consistent antitumor effects, as demonstrated in murine models of syngenic colon carcinoma and melanoma.[217] In addition, IMO-2055 has recently been shown to impair EGFR signaling and to synergize—in an immune system-independent fashion—with cetuximab for the treatment of human colon carcinoma xenografts.[218,219] In patients with advanced NSCLC, the use of IMO-2055 in combination with gemcitabine + carboplatin has been associated with no hematological toxicity and signs of efficacy (ref. 220 and Annual AACR Meeting 2006, Abstract #P3–022). Subsequent trials have investigated the safety and efficacy of IMO-2055 as a single agent in patients with recurrent/metastatic renal clear cell carcinoma (NCT00729053), combined with cetuximab + FOLFIRI (folinic acid + 5-fluorouracil + irinotecan) in colorectal cancer patients (NCT00719199) or with cetuximab + 5-fluorouracil + cisplatin in subjects with recurrent/metastatic HNSCC (NCT01360827), and in association with the EGFR inhibitor erlotinib[221] + bevacizumab (an anti-angiogenic monoclonal antibody) in NSCLC patients (NCT00633529). The results of these studies, however, have not yet been disclosed. Today, official sources list one single study based on IMO-2055 as active, in which the drug is evaluated (in combination with cetuximab) for the treatment of cetuximab-naïve subjects with recurrent/metastatic HNSCC (NCT01040832) (source www.clinicaltrials.gov).

The immunostimulatory sequence (ISS) 1018 is a single-stranded, 22 bp-long, phosphorothioate oligonucleotide prepared by standard solid-phase chemistry techniques (sequence 5'-TGA CTGTGAACGTTCGAGATGA-3').[222] The administration of 1018 ISS to simian immunodeficiency virus (SIV)-infected macaques reportedly results in massive activation of plasmacytoid DCs, IL-12 production and accumulation of IFNγ-secreting SIV-specific T cells.[223] Along similar lines, ISS 1018 has been shown to trigger an IL-12-dependent cytotoxic immune response that efficiently prevents the growth of spontaneous B-cell lymphomas in SJL mice.[224] Following early clinical trials that demonstrated its safety,[225] ISS 1018 has been extensively tested as part of a vaccine against the hepatitis B virus (HEPLISAV™).[226] Moreover, ISS 1018 has been shown to be safe and moderately effective in follicular non-Hodgkin lymphoma patients treated with the anti-CD20 antibody rituximab (NCT00251394).[227-229] Of note, the results of Phase I clinical trial in which ISS 1018 was combined with irinotecan + cetuximab to treat metastatic colorectal carcinoma patients have not been released yet (NCT00403052) (source www.clinicaltrials.gov).

MGN-1703 and MGN-1706 belong to the class of DNA-based double stem-loop immunomodulators (dSLIMs), i.e., IMOs that contain a phosphodiester, rather than a phosphorothioate, backbone and fold in a dumbbell-like covalently-closed structure.[230] dSLIMs stimulate cultured peripheral blood mononuclear cells (PBMCs) from healthy donors to secrete Th1 cytokines, including IFNγ, IL-2 and IL-12.[230] Moreover, dSLIMs have been shown to enhance the antileukemic effects of peptide vaccines combined with the granulocyte monocyte colony stimulating factor (GM-CSF), in vivo.[231] Seventeen metastatic colorectal cancer patients receiving dSLIMs in combination with chemotherapy and a peptide vaccine developed no severe toxicity, yet in this setting the adjuvant failed to boost anticancer immune responses.[232] More recently, the safety of MGN-1703 has been confirmed in patients with advanced colorectal carcinoma showing disease control after first-line standard chemotherapy (see refs. 233, 234 and Annual ASCO Meeting 2011, Abstract 618; Annual ASCO Meeting 2012, Abstract 633). This study, which intends to evaluate not only the safety but also the efficacy of MGN-1703 as a single agent for maintenance therapy, is still ongoing (NCT01208194) (source www.clinicaltrials.gov).

The clinical development of additional TLR9 agonists including AVE0675, IMO-2125, QAX-935 and SAR-21609 and SD-101, which until recently were being tested for the treatment of asthma/allergy or hepatitis C virus infection,[16,176,177]

has been discontinued. On the contrary, DIMS0150 (kappaproct) is under investigation in a Phase III study involving patients with ulcerative colitis (NCT01493960) (source www.clinicaltrials.gov).

Table 5 summarizes recent clinical progress of experimental TLR9 agonists for oncological indications.

Others

TLR5 is mainly expressed at the surface of intestinal epithelial cells, monocyte, macrophages and DCs.[16] The only known natural ligand for TLR5 is flagellin, a component of bacterial flagella.[235,236] In particular, TLR5 recognizes a short, highly conserved domain of flagellin that participates in intermolecular interactions within flagellar protofilaments and hence is required for bacterial motility.[237] Recent crystallographic data indicate that TLR5 exhibits a peculiar ligand binding mode, even though it dimerizes following ligand binding similar to all other TLRs.[236,238]

CBLB502 is a peptide derivative of *Salmonella enterica* flagellin that has been developed as a TLR5 agonist.[239] Similar to full-length flagellin, CBLB502 potently activates NFκB in vitro, hence exerting consistent anti-apoptotic effects.[239] In line with this notion, CBLB502 attenuates injury in a murine model of ischemic renal failure[240] and protects mice as well as primates from the life-threatening consequences of total body irradiation.[239,241] Importantly, CBLB502 has been shown not to interfere with tumor radiosensitivity, at least in mice models,[239] suggesting that CBLB502 might be employed as a radioprotective agent in cancer patients undergoing radiation therapy. Clinical trials that would evaluate this possibility have not yet been initiated, but the safety and efficacy of CBLB502 (used as a standalone agent) are being investigated in a single Phase I study enrolling patients with advanced unresectable solid tumors (NCT01527136). Intriguingly, full-length flagellin appears to inhibit the growth of immunogenic cancers in mice, but only if administered 8–10 d after tumor implantation, whereas it would exert pro-tumor effects if administered earlier.[242] Although a few trials have recently investigated the efficacy of flagellin fused to a portion of the influenza virus matrix protein 2 (VAX102) as a vaccine against flu,[243,244] when this paper was submitted (April 2012), no clinical trials were investigating the possibility to use full

Table 5. Clinical trials evaluating TLR9 agonists in oncological indications*

Compound	Indication	Trials	Phase	Status	Notes	Ref.
Agatolimod	B-cell lymphoma	1	II	Recruiting	Combined with radiotherapy	NCT00880581
	Bladder cancer	2	I	Enrolling by invitation	Combined with BCG, MPL and recMAGE-A3	NCT01498172
			II	Recruiting	Combined with MPL and recMAGE-A3	NCT01435356
	Breast cancer	2	I-II	Active, not recruiting	Combined with MPL, a HER2-based vaccine and lapatinib	NCT00952692
			II	Recruiting	Combined with trastuzumab	NCT00824733
	Esophageal cancer	1	I-II	Recruiting	Combined with a multipeptide vaccine	NCT00669292
	Melanoma	4	0	Recruiting	Combined with MPL and recMAGE-A3	NCT01425749
			I		Combined with MPL	NCT01149343
			II		Combined with IL-2, MPL and recMAGE-A3	NCT01266603
			II		Combined with MPL, recMAGE-A3 ± Hiltonol	NCT01437605
	Multiple myeloma	1	I	Recruiting	Combined with MPL and recMAGE-A3	Recruiting
	NY-ESO-1-expressing tumors	1	I-II	Recruiting	Combined with a multipeptide vaccine ± cyclophosphamide	NCT00819806
GNKG168	B-CLL	1	I	Recruiting	As single agent	NCT01035216
IMO-2055	Colorectal cancer	1	I	Terminated	Combined with cetuximab and FOLFIRI	NCT00719199
	Head and neck carcinoma	2	I	Terminated	Combined with 5-FU, cisplatin and cetuximab	NCT01360827
			II	Active, not recruiting	Combined with cetuximab	NCT01040832
	NSCLC	1	I	Completed	Combined with erlotinib and bevacizumab	NCT00633529
	RCC	1	II	Completed	As single agent	NCT00729053
MGN-1703	Colorectal cancer	1	II	Recruiting	As single agent	NCT01208194

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; FOLFIRI, folinic acid, 5-FU, irinotecan; 5-FU, fluorouracil; IL-2, interleukin 2; MPL, monophosphoryl lipid A; NSCLC, non-small cell lung carcinoma; RCC, renal cell carcinoma; recMAGE-A3, recombinant MAGE-A3. *Started after January, 1st 2008.

length flagellin as an adjuvant for anticancer therapy (source www.clinicaltrials.gov).

Cadi-05 (Immuvac), which consists of autoclaved *Mycobacterium indicus pranii* (also known as Mycobacterium w), has been originally developed as a vaccine against leprosy, an indication for which it is nowadays approved by FDA.[245] Cadi-05 functions as a polyTLR agonist and has been shown to trigger potent anticancer immune responses in vivo, featuring increased levels of circulating IFNγ as well as the accumulation of tumor antigen-specific $CD4^+$ and $CD8^+$ T cells.[246,247] In immunocompetent mouse models of myeloma, thymoma and melanoma, the intravenous administration of Cadi-05 (as a single agent) a few days after tumor implantation was sufficient to suppress neoplastic growth.[246,247] The anticancer properties of Cadi-05 mainly rely on IFNγ, as they were abrogated in *Ifng*$^{-/-}$ mice.[247] The use of Cadi-05 as an adjuvant seems promising, at least according to early reports on invasive bladder cancer patients treated with radiotherapy[248] and NSCLC patients receiving radiotherapy in combination with cisplatin and etoposide.[249] Subsequent studies have confirmed the safety and efficacy of Cadi-05 in this latter indication (ref. 250 and Annual AACR Meeting 2006, Abstract #P3–022) as well as in other oncological settings, including advanced solid tumors refractory to therapy (Annual ASCO Meeting 2008, Abstract 14019). In addition, Cadi-05 has recently been investigated as a standalone agent for the treatment of superficial transitional cell carcinomas of the bladder (NCT00694798), as a single agent in Stage III-IV melanoma patients (NCT00675727), combined with docetaxel in patients affected by hormone refractory metastatic prostate cancer (NCT00525408), and associated to paclitaxel + cisplatin in NSCLC patients (NCT00680940). The results of these studies, however, are still unpublished. At the time of submission of this Trial Watch (April 2012), only one Phase II clinical trial was ongoing to evaluate the efficacy of Cadi-05 in patients bearing superficial transitional cell carcinomas of the bladder (NCT00694915) (source www.clinicaltrials.gov).

Similar to Cadi-05, IMM-101 consists of inactivated bacteria, and more precisely of heat-killed *Mycobacterium obuense*.[251] IMM-101 has been shown to indirectly activate γδ T cells (via a particular subset of myeloid DCs), resulting in the secretion of Th1 cytokines including IFNγ and TNFα and in the activation of antitumor immune responses.[252] Although the preclinical characterization of IMM-101 is scarce, many other preparations that are similar in concept (including SRL172, which consists of heat-inactivated *Mycobacterium vaccae*) have been extensively investigated in humans for a variety of diseases, including cancer.[253] SRL172 has been associated with minimal toxicity, potent immunostimulatory effects and—in some instances—a promising anticancer profile.[254-256] These observations prompted for a Phase I clinical study to evaluate IMM-101 in stage III/IV melanoma patients (NCT01308762), which confirmed IMM-101 to be safe and potentially efficient.[257] Today, the clinical benefits of SRL172 are being investigated in a few trials on cancer-unrelated indications (mainly tuberculosis). On the contrary, IMM-101 is being tested—combined with radiation therapy—in patients with previously treated colorectal cancer (NCT01539824), and—in combination with gemcitabine—in patients affected by advanced pancreatic cancer (NCT01303172) (source www.clinicaltrials.gov).

Table 6 summarizes recent clinical progress of experimental TLR5 and polyTLR agonists for oncological indications.

Concluding Remarks

A vast amount of preclinical and clinical data indicate that TLR agonists exert potent immunostimulatory functions, in vivo. This said, with the notable exceptions of BCG, MPL and imiquimod (which are approved by FDA and the European Medicines Agency) and perhaps picibanil (which is approved by the Japanese Ministry of Health and Welfare), the clinical progress of TLR agonists for use as standalone agents or adjuvants in cancer therapy appears to have reached an impasse. The development of many TLR agonists has been discontinued due to a lack of efficacy in Phase III studies, and the number of clinical trials currently testing this rather large class of compounds (including no less than 30 distinct molecules) for oncological indications is small (< 50 trials, all phases confounded, excluding BGC-, MPL-, imiquimod and picibanil-based studies). This points to some decline in the interest of researchers and clinicians for TLR agonists, at least in the oncological setting. One element that has surely contributed to (though perhaps not entirely determined)

Table 6. Clinical trials evaluating TLR5 and polyTLR agonists in oncological indications*

Compound	Indication	Trials	Phase	Status	Notes	Ref.
CBLB502	Adult solid tumors	1	I	Recruiting	As single agent	NCT01527136
CADI-05	Bladder cancer	2	I	Completed	As single agent	NCT00694798
			II	Active, not recruiting	As single agent	NCT00694915
	Melanoma	1	I/II	Terminated	As single agent	NCT00675727
	NSCLC	1	II	Completed	Combined with cisplatin and paclitaxel	NCT00680940
	Prostate cancer	1	II	Terminated	Combined with docetaxel	NCT00525408
IMM-101	Colorectal cancer	1	II	Recruiting	Combined with SBRT	NCT01539824
	Melanoma	2	I	Completed	As single agent	NCT01308762
			I/II	Enrolling by invitation	As single agent	NCT01559818
	Pancreatic cancer	1	II	Recruiting	Combined with gemcitabine	NCT01303172

Abbreviations: NSCLC, non-small cell lung carcinoma; SBRT, stereotactic body radiotherapy. *Started after January, 1st 2008.

this decline is the limited availability of clinical grade TLR agonists, pushing academic investigators to prefer surrogate compounds, such as clinically approved prophylactic vaccines.[258,259] Our hope is that the results of ongoing clinical trials will invert this tendency and eventually lead to the development (and approval) of optimal immunochemotherapeutic regimens that fully exploit the immunostimulatory potential of TLR agonists against cancer.

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