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REVIEW

Targeting pattern recognition receptors in cancer immunotherapy

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Abstract Pattern recognition receptors (PRRs) are known for many years for their role in the recognition of microbial products and the subsequent activation of the immune system. The 2011 Nobel Prize for medicine indeed rewarded J. Hoffmann/B. Beutler and R. Steinman for their revolutionary findings concerning the activation of the immune system, thus stressing the significance of understanding the mechanisms of activation of the innate immunity. Such immunostimulatory activities are of major interest in the context of cancer to induce long-term antitumoral responses. Ligands for the toll-like receptors (TLRs), a well-known family of PRR, have been shown to have antitumoral activities in several cancers. Those ligands are now undergoing extensive clinical investigations both as immunostimulant molecules and as adjuvant along with vaccines. However, when considering the use of these ligands in tumor therapy, one shall consider the potential effect on the tumor cells themselves as well as on the entire organism. Recent data indeed demonstrate that TLR activation in tumor cells could trigger both pro- or antitumoral effect depending on the context. This review discusses this balance between the intrinsic activation of PRR in tumor cells and the extrinsic microenvironment activation in term of overall effect of PRR ligands on tumor development. We review recent advances in the field and underline appealing prospects for clinical development of PRR agonists in the light of our current knowledge on their expression and activation.

Keywords TLR · RLR · Cancer · Immunotherapy · Vaccine

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Background

Cancer immunotherapy consists of approaches that mobilize components of the immune system as cancer treatment (reviewed in [1]). The history of cancer immunotherapy dates back to Coley's original observation of the late nineteenth century of antitumor effects from a bacterial concoction [2]. Activation of the immune system was proposed as the mechanism leading to the regression of these measurable tumors. These interpretations are now reinforced by the successful use of purified microbial extracts in cancer treatment as well as the understanding of their mechanism of action.

The recognition of pathogens is the key to understand the activation of immune response not only by infectious non-self agents but also by endogenous "danger" signals. Our understanding of innate immunity has led to the discovery of innate sensors or pattern recognition receptor (PRR) being able to recognize pathogen-associated molecular patterns (PAMPs) of all class of microorganisms [3]. Such PRR consists of four families including toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), and DNA sensors (reviewed in [4–6]). Those receptors were recently involved in the sensing of endogenous ligands, referred as danger-associated molecular patterns (DAMP), released by cells in non-infectious conditions such as stress, injury, or cell death [7].

Localization of those receptors at the membranes (TLR) or in the cytosol (RLR, NLR, and DNA sensors) is intimately linked to the nature of the DAMP/PAMP they sense [6]. For instance, TLRs (1/2/4/5/6) involved in bacteria and fungi recognition are expressed at the cell surface, with TLRs specialized in virus recognition through sensing of nucleic acids (TLR3/7/8/9) are located within the endosomes (summarized in Table 1, [6, 8]). In addition to the TLRs, two recently described families of cytosolic innate sensors are involved in virus recognition: (a) the RNA helicases [4] and (b) the cytosolic DNA receptors (DAI, AIM2, and IFI16/p204) [5]. The cytoplasmic RNA helicases, RIG-I, and melanoma differentiation-associated gene 5 (MDA5) and DDX1/DDX21/DHX36 complex [9], sense RNA or DNA viruses that replicate in the cytosol via an RNA intermediate. DNA sensors would be involved in the detection of DNA from viruses and intracellular bacteria [herpes simplex virus (HSV)-1, *Listeria monocytogenes*, *Francisella tularensis*, and vesicular stomatitis virus (VSV)] [10]. Finally, NLRs include NOD1/2 involved in bacterial recognition as well as the NLRP family members that sense various PAMP and DAMP [11].

The activation of these pathways relies on various adaptors downstream of the different PRR: myeloid differentiation factor 88 (MYD88) and toll/IL-1 receptor (TIR) domain-containing adaptor inducing interferon β (TRIF) for TLR, mitochondrial antiviral signaling (MAVS) (also known as IPS1/CARDIF/VISA) for RNA helicases, stimulator of interferon genes for most DNA sensors [10], and apoptosis-associated speck-like protein for NLRP.

Triggering of those PRRs results in the induction of proinflammatory cytokines, type I interferon (IFN), and chemokines that altogether participate in the establishment of an adjusted innate and adaptive immune response. Type I IFNs, which have a key role in antiviral immunity, are produced by most cell types through the RNA helicase pathway in response to virus infection [12, 13]. In contrast, plasmacytoid dendritic cells (pDCs), the predominant source of type I IFNα in vivo, are triggered via TLR7 and TLR9 particularly expressed on those cells. As a general theme, membrane-bound PRRs recruit members of the interleukin-1 receptor-associated kinase and tumor necrosis factor receptor-associated factor families to stimulate gene expression via activation of transcription factors, mostly NF-κB, the interferon regulatory factors (IRFs), and MAP kinases. In contrast, the activation of NLRP-1 and -3 [14–19], as well as the DNA sensors AIM2 [14, 20–23], triggers the formation of a multimolecular complex termed inflammasome, which leads to the proteolytic cleavage of pro-interleukin (IL)-1/IL-18 after microbial, DAMPs, or genomic DNA sensing (reviewed in [24]).

Besides cytokine/chemokine production, PRR activation provides essential requirements for the initiation of T cell response: enhancement of antigen uptake, processing, and presentation (through the upregulation of major histocompatibility complex (MHC) and costimulatory molecules expression) by antigen-presenting cells (APCs) including dendritic cells (DCs), followed by migration of those DCs after modulation of chemokine receptor expression. All those events contribute to the activation of antigen-specific T cell [25].

Therefore, the engagement of the innate system via PRR on both immune and tumor cells combined with the triggering of adaptive immune responses provides us with interesting prospects regarding the treatment of cancers. This review discusses the advantages of associating PRR ligands, with a specific focus on TLR and RLR, to conventional therapies in order to promote direct antitumor activity as well as antitumor immune response. Although several mouse models support such therapeutic approach, it is worth remembering that differences in expression and signaling capacity between murine and human PRRs are likely to affect the efficacy for controlling cancer cells. We have therefore essentially focused the discussion on human PRRs.

Toll-like receptors

TLR belongs to the most largely described family of PRRs. *Toll* was initially discovered in the fruit fly where it was shown to mediate Drosophila dorsoventral embryonic development and innate immune response [26, 27]. The first mammalian homolog of *Toll*, now known as TLR4, was reported in 1997 by Medzhitov et al. [28]. TLR4 was shown to be involved in the recognition of lipopolysaccharide (LPS), a major cell wall component of gram-negative bacteria. Ten human TLRs have been identified since then. TLRs are type I transmembrane proteins with an extracellular domain consisting of leucine-rich repeats for ligand binding and a cytoplasmic TIR domain for downstream signaling. TLR signaling involves a family of five TIR domain-containing adaptor molecules (MYD88, TRIF, TRAM, MAL, and SARM) [3, 6]. All TLRs require MYD88 for signaling, except TLR3, which uses exclusively TRIF and TLR4 that recruits both adaptors to initiate different signaling pathways. Moreover, besides MYD88 and TRIF, TLR4 uses as well the adaptor molecule MAL for MYD88-dependent NF-κB activation and TRAM to drive TRIF-dependent type I IFN induction via IRF activation. Ensuring that these responses are tightly regulated in order to maintain homeostasis and prevent overwhelming immune activation is thereby crucial and relies on many TLR regulators described over the years [3, 6].

TLR1/2/4/5/6 are mostly involved in bacteria and fungi PAMP recognition (Table 1) [6]. The receptors for nucleic acid-based PAMPs, i.e., TLR3, -7/8, and -9, recognize

Table 1 Cellular expression and ligands of TLR and RLR

PRR	Cellular expression		Ligands		Reference
	Immune cells	Non-immune cells	PAMP	DAMP	
MDA5	Ubiquitous	Ubiquitous	Long dsRNA [polyI:C], EMCV		[72]
RIG-I	Ubiquitous	Ubiquitous	5′triphosphate dsRNA, short Poly[I:C], RNA viruses (NDV, VSV, SeV, flu ΔNS1, HCV, JEV)		[70, 71]
TLR1/2	Monocyte, mDC, B cell, NK, neutrophil, basophil	Ubiquitous	LAM, PGN, GPI (*Toxoplasma gondii*), LTA, triacyl lipopeptide, zymosan		[6, 8, 31, 32]
TLR2/6	Monocyte, mastocytes, mDC	Epithelial cell, neural cell, MSC, endothelial cell	LTA, diacyl lipopeptide		[6, 47–49]
TLR3	mDC	Epithelial cell, neural cell, MSC	RNA virus (WNV, RSV, MCMV), synthetic dsRNA (Poly[I:C], poly [A:U])		[32, 80]
TLR4	Monocyts, macrophages, mDC, mastocytes, basophil	Renal cell, hepatic cell, keratinocytes, MSC, endothelial cell	LPS, viral proteins (HIV, VSV, RSV, retroviruses)	HSP, fibronectin, hyaluronic acid, fibrinogen	[28, 45–49]
TLR5	mDC, monocyte, NK, T cell	Gastric epithelial cell, keratinocytes, MSC	Flagellin		[45, 46, 48]
TLR7	pDC, B cell, esosinophil		RNA virus (influenza), synthetic ssRNA, imidazoquinoline (R848/CL097/imiquimod)		[8, 171–173, 176]
TLR8	mDC, T and B cell, monocyte		Synthetic ssRNA (AU-rich) imidazoquinoline (R848/CL075)		[8, 171–174]
TLR9	pDC, B cell, basophil, eosinophil	Epithelial cell, keratinocytes, MSC, endothelial cell	Unmethylated CpG dsDNA, dsDNA virus (HSV, MCMV)	DNA/LL-37	[32–34, 44, 57, 210–214, 219, 221–224, 230]
TLR10	pDC, neutrophil, B cell, basophil		Unknown		[8, 37–39]

mDC myeloid DC, *NK* natural killer cell, *pDC* plasmacytoid DC, *MSC* mesenchymal stem cell, *LTA* lipotechoic acid, *LAM* lipoarabinomannan, *PGN* peptidoglycan

double stranded (ds), single stranded (ss) viral RNA, and unmethylated CpG DNA, respectively. Ligand for hTLR10 has not yet been described. In contrast with mice [29, 30], the human TLR11 gene is not functional. TLR activation results in transcription of proinflammatory cytokines such as TNFα, IL-1β, IL-12, and IL-6, and in some cases, in type I IFN genes. The cytokine induction pattern is determined by the type of TLR activated, the nature of the ligand, and activated cell.

Expression of TLRs was first documented in immune cells. TLR1 and 6 are ubiquitously expressed in human leukocytes [8, 31, 32]. TLR2/4/5 are restricted to myelomonocytic cells [32–34]. TLR3 is expressed in resting cells by mDC [32] and by some subsets of human B cells [35] and is upregulated in LPS-stimulated monocytes [36]. pDC and B cell specifically express TLR-7, 9, and -10 [8, 37–39]. TLR1/2/6 triggering by bacterial PAMP induce a proinflammatory response in myelomonocytic cells (Table 1). In contrast, triggering of pDC by nucleic acid PAMP induces production of type I IFN, critical for innate antiviral immunity and development of an adequate adaptive immune response [39]. Type I IFN production relies on the activation of transcription factors, especially IRF-3 and -7. TLR3/4 activate IRF3 via TRIF [40, 41], while TLR7/9 induce a response exclusively via MYD88 [42, 43]. In humans, this later response occurs mostly in pDC and depends on IRF7 rather than on IRF3. Unlike other type of cells, pDC constitutively expresses high levels of IRF7 and are therefore preprogrammed to quickly produce high levels of IFNα [42, 43].

While TLR are primarily expressed on immune cells, they have been described in human keratinocytes [44, 45], epithelial cells from the intestinal, urogenital, and respiratory tracts [46], endothelial cells [47], mesenchymal stem cells [48], and various neural cells [49]. In these tissues, they are likely to provide a first line of innate antimicrobial defense. Moreover, several studies have shown that TLRs, as other PRR indeed [50], are capable of controlling intestinal epithelial homeostasis through proliferation and protection from injury [51, 52]. Hasan et al. reported that stimulation of TLRs induces, via MYD88 recruitment, cell cycle entry and progression in fibroblasts [37].

TLR have garnered an extraordinary amount of interest in cancer research due to their role in tumor progression. Besides the extrinsic role of TLR on cells from the tumor microenvironment (immune cells, fibroblasts, and endothelial cells), the intrinsic role of TLR on tumor cells needs to be considered as well. The importance of TLR in host response to tumors became evident as polymorphisms in TLR genes were associated to susceptibility not only to infections but also to cancers [53]. Increasing evidences suggest that TLR might play a dual role in cancer progression (reviewed in [54]). Activation of different TLR might exhibit the exact opposite outcome, antitumor or protumor effects. For example, TLR9 activation was shown to cause breast tumor cell proliferation and increased invasiveness [55] while it inhibited in vitro and in vivo cell proliferation and induced cell death in neuroblastoma cells [56]. Furthermore, TLR expression is modulated on tumor cells, where they might influence tumor growth and host innate immune response. TLR9 expression was shown for instance to be downregulated in head and neck and cervical cancers [57] and upregulated in breast cancer with poorly differentiated phenotype [58]. Increase of TLR3 expression in a percentage of breast cancer patients was for instance associated to a high response rate to synthetic dsRNA, polyadenylic–polyuridylic (Poly[A:U]) treatment [59]. The expression of TLR1 to TLR10 is induced in lymphocytes and macrophages from healthy volunteers following exposure to anticancer agents that induce the p53 network suggesting that these immune receptors can be modulated by DNA metabolic stress [60].

Manipulating TLR signaling in the context of cancer thereby requires a special care particularly with regard to the effect of TLR activation on tumor cells as well as the tumor microenvironment. Moreover, as TLR expression differs between mouse and human, results from preclinical study should be taken very carefully for transfer towards clinical trials.

RNA helicases

RIG-I/DDX58 and MDA5/RH116 (also known as IFIH1 and Helicard) are ubiquitously expressed cytoplasmic sensors involved in RNA detection [4, 13, 61], called RIG-like receptors. These helicases belong to the DExD/H box RNA helicase family including a large number of proteins involved in RNA metabolism [62]. They are able to bind and unwind ssRNA through their helicase domain and signal via their caspase recruitment domains (CARD). Activation of these helicases induces their recruitment to the adaptor protein MAVS located to the mitochondria [63–66]. MAVS relays signals via phosphorylation cascade and nuclear translocation of transcription factors such as NF-κB and IRF3 to coordinate type I IFN production mostly, as well as secretion of proinflammatory cytokines. The third member of the family, laboratory of genetics and physiology 2 (LGP2), lacks CARD domains and acts as a regulator of RIG-I and MDA5 signaling [67–69]. These helicases are involved in virus detection through sensing of their RNA genome. Both helicases sense RNA molecules but with distinct specificity; RIG-I sense 5′-triphosphate (5′tri-P) moiety on RNA backbone [70, 71] while MDA5 is more specific of long dsRNA mimicked in vitro using synthetic polyinosinic–polycytidylic acid (Poly[I:C]) [72] (reviewed in [73]). In that context, RIG-I is essential for the production of type I IFN in response to in vitro-transcribed RNA and RNA viruses including paramyxoviruses (SeV, NDV, and VSV), influenza virus, and Japanese encephalitis

virus, whereas MDA5 is critical for Poly[I:C] and picornavirus (EMCV) detection [72]. Phenotypic analyses of a RIG-I deficient mouse model further showed that, besides its involvement in type I IFN induction, RIG-I is an essential negative regulator of physiological myeloid cell differentiation [74]. Besides their role in virus sensing, RLRs are also involved in cancer development. MDA5 was indeed initially found and named for its ability to inhibit colony formation in human melanoma cells [61].

Other DEAD-box proteins were also recently involved in PAMP sensing. Liu and colleagues showed that DHX36 and DHX9 interact respectively with CpG-A and CpG-B oligonucleotides (ODN) and that cells deficient for those helicases display reduced cytokine production [75]. DDX36 and DHX6 thus represent the non-TLR9 MYD88-dependent pathway previously described in pDC [76–78]. More recently, the same group identified DDX1, DDX21, and DHX36 as part of a complex that detects Poly[I:C] in murine splenic DC and signals through TRIF. TRIF$^{(-/-)}$ cells were previously shown to be more deeply impaired in their response to Poly[I:C] than TLR3$^{(-/-)}$ cells suggesting a non-TLR3 TRIF-dependent mechanism of Poly[I:C] sensing [79]. Four systems have thus been reported for Poly[I:C] recognition: (a) the endosomal TLR3-TRIF pathways crucial in epithelial cells [79–81], (b) the RIG-I/MDA5/MAVS mitochondrial pathway that play an important role in fibroblasts and DC [12, 82], (c) the protein kinase R pathway, and (d) this DDX/TRIF pathway in murine DC [9].

The engagement of the innate system via PRR in combination with other anticancer agents therefore provides us with a very exciting prospect of the development of new approaches to treat malignancies. As above mentioned, one shall indeed consider several aspects when thinking about the use of such ligands in cancer immunotherapy: (a) the intrinsic effect on the tumor cells as well as (b) the extrinsic role of TLR activation in cells from the microenvironment and especially immune cells and (c) the adjuvant activity when combined to vaccine. Indeed, human and mice tumors often contain infiltrating immune cells. The potential protective role of these cells has been demonstrated in colorectal cancer patients [83]. Moreover, the development of cancers is favored by immunosuppression in human or immunodeficiency in mice, suggesting a need for reactivation of immune activation in cancer therapy. This review will discuss all these aspects in the prospect of using RLR and TLR ligands, divided in bacterial TLR ligands, TLR3, TLR7/8, and TLR9 ligands, to treat cancer.

Bacterial TLR ligands

As mentioned in the "Background" section, systemic antitumor effects of bacterial infections were reported over a century ago. In retrospect, antitumor effects were probably due to PRR activation, and certainly mostly TLR or NLR, by endotoxins and DNA contained in this heat-killed bacteria preparation. Various bacterial lipopolysaccharides and glycoproteins have been of interest in cancer treatment since Coley's observations. Examples include Bacillus Calmette-Guerin (BCG), whose first published use in cancer dates back to 1935, and *Corynebacterium parvum* (lung cancer), streptococcal preparation OK432 (carcinomatous pleural effusions), biostim®—glycoprotein extract from *Klebsiella pneumoniae* (advanced colorectal cancer), and bestatin® from *Streptomyces olivoreticuli* (leukemia and solid tumors), about 50 years later. However, BCG is the only one to have received regulatory approval. Identification of the recognition of bacteria by TLRs led to the development of purified TLR ligands now in use in clinical trials mostly for their adjuvant activity. As many clinical trials are currently using bacterial TLR ligands as monotherapy or adjuvants in cancer therapy, Table 2 only lists phase III clinical trials.

Bacterial TLR: ligands and cellular expression

TLR ligands involved in bacterial recognition include TLR1/2/4/5/6. mTLR11 was shown to sense uropathogenic *Escherichia coli* bacteria and profiling-like protein from *Toxoplasma gondii* [29, 30]; however, its human equivalent contains several stop codons and does not code for a full-length protein [84]. TLR2 acts as heterodimer with TLR1 or TLR6 and senses PAMP from bacteria (lipoarabinomannan, lipoteichoic acid, peptidoglycan, diacyl, or triacyl lipopeptide), fungi (zymosan, together with dectin 1), and parasite (GPI from *T. gondii*) (Table 1) [6]. As mentioned earlier, TLR4 recognizes LPS, a cell wall component of gram-negative bacteria, and its minimal unit the lipid A. TLR4 was shown too to bind viral proteins from RSV [85, 86], VSV [87], retroviruses [88, 89], and HIV [90]. Moreover, TLR4 was shown to sense self-DAMP liberated during tissue injury (hyaluronan, fibronectin, and heat shock proteins 60 and 70 [30]) or upon chemotherapeutic treatment (high mobility group box-1 (HMGB1); [91]).

TLR1 and 6 are ubiquitously expressed in human leukocytes [8, 31, 32]. TLR2, TLR4, and TLR5 are restricted to myelomonocytic cells, such as mDC, monocytes, neutrophils, and basophils [32–34]. Besides immune cells, such TLRs are also expressed on endothelial cells (TLR2/4 [47]), mesenchymal cells (TLR2/4/5 [48]), neural cells (TLR2/4 [49]), keratinocytes (TLR4/5 [45]), and epithelial cells from intestinal, urogenital, and respiratory tracts. TLR2/4 triggering by bacterial PAMP induce a proinflammatory response in myelomonocytic cells (Table 1). Signaling in epithelial cells via TLR4 and TLR5, in concert with TLR signaling in leukocytes, does participate to the development of intestinal inflammation [46]. Upon ligand binding and endocytosis, activated TLR4 might result as well in type I IFN secretion [92].

Table 2 Bacterial TLR ligands in phase III clinical trials

Effect[a]	Clinical phase	Status[b]	Designation	Cancer type	Reference
D	Phase III	A/R-2011	BCG +/− Gefitinib	Bladder cancer	NCT00352079
D	Phase III	R	Hyperthermia, BCG, and mitomycine C	Bladder cancer	NCT01094964, NCT00384891, NCT00974818
D	Phase III	R-2010	BCG vs uracil-tegafur	Bladder cancer	NCT01082510
D	Phase II/III	C-2005	BCG and IFNα	Bladder cancer	NCT00330707
D	Phase III	W	EN3348 vs BCG	Bladder cancer	NCT01284205
A	Phase III	C	HPV 16/18 L1 VLP AS04 vaccine	Cervical neoplasia and cancer	NCT00316706, NCT003344032, NCT00426361, NCT00485732, NCT00345878, NCT00492544, NCT00122681
A	Phase III	A	HPV 16/18 L1 VLP AS04 vaccine in various cohorts	Cervical neoplasia and cancer	NCT0029047
A	Phase III	C	HPV vaccine with Engerix or Twinrix	Cervical neoplasia and cancer	NCT00652938, NCT00578227
A	Phase III	R-2007	MAGE-A3/AS15 vaccine	NSCLC	NCT00480025
A	Phase III	R-2008	MAGE-A3/AS15 vaccine	Melanoma	NCT00796445
A	Phase III	R	BLP25 vs placebo	NSCLC	NCT00409188, NCT01015443
A	Phase III	T	BLP25 and hormonal treatment	Breast cancer	NCT00925548
A	Phase III	C	Detox-B vs STn-KLH, KLH, or cyclophosphamide	Breast cancer	NCT00003638

www.clinicaltrial.gov

[a] Effect: *A* adjuvant, *D* direct; effect of therapeutic combination

[b] Clinical trial status; *A* active, *R* recruiting, *C* completed, *W* withdrawn, *T* terminated; with completed date or starting date for active and recruiting trials

Dual role of intrinsic TLR triggering on tumor cells

Hundreds of single nucleotide polymorphisms (SNPs) have been identified in TLRs but their functional consequences are largely unknown. Many associations have been reported between TLR polymorphisms and infectious disease. For instance SNPs in either TLR2/4/5, which are expressed in epithelial cells and involved in bacterial recognition, result in increased risk of sepsis and bacterial infection (reviewed in [53]). Recent analysis of data compiled from several studies showed that in contrast to initial data, no overall association exists between SNPs in the TLR4 and TLR6-1-10 loci and risk of prostate cancer [93]. It seems that in most case, the infection and chronic inflammation most likely mediates the increased risk of cancer development [53].

As for other PRR, expression of TLR2/4/5 is upregulated in various tumor epithelial cells. The upregulation of TLR2/4/5 in gastric epithelial cells from patients with gastric dysplasia and carcinoma suggests that these receptors may play a role in adenocarcinoma development [94]. Similarly, TLR4 was shown to be upregulated in human ovarian [95], colon [96], and head and neck tumor cells [97]. TLR triggering in such epithelium does induce inflammation that has indeed been linked to tumor cell resistance to chemotherapies and cell growth. Triggering TLR4–MYD88 pathway in human ovarian [95] and head and neck tumor cells [97] promoted chemoresistance and tumor growth in vitro [95, 97]. In addition, the MYD88 expression in ovarian cancer tissues correlated with a lower patients' progression-free survival [95]. Pro-tumoral activity of TLR4 signaling was shown to be mediated by both intrinsic and extrinsic mechanisms. LPS triggering of TLR4 directly increases tumor cell proliferation [97]. In parallel, tumor growth was favored via synthesis of soluble factors (IL-6, inducible nitric oxide synthase, IL-12) favoring a pro-tumoral microenvironment. These factors have indeed been reported to increase resistance of tumor cells to cytotoxic attack by both T and NK cells [95, 96] and to favor the development of myeloid-derived suppressor cells. Similarly, TLR2 stimulation by *L. monocytogenes* promoted tumor growth via the production of immunosuppressive molecules such as IL-6 or nitric oxide [98]. In contrast, intrinsic activation of TLR5 by flagellin inhibited proliferation of breast cancer cells and tumor growth in xenograft model [99]. Altogether, the activation of different TLRs might display completely different outcome.

Triggering of bacterial TLR ligands on immune cells for antitumoral activity

Nowadays, live, attenuated culture preparation of the BCG strain of *Mycobacterium bovis* [TICE® (Organon Teknika

Corp) and PACIS® (BioChem Pharma)] is currently in use for cancer therapy. They were granted FDA approval over 30 years ago for intravesical treatment of in situ carcinoma (CIS) of the urinary bladder and primary or recurrent state papillary tumors (stage I, Ta, and T1). Several multicenter clinical trials showed increased benefits when compared to intravesical doxorubicin treatment based on complete response rate for patients with CIS (70% vs 34% [100], 71% vs 54% [101]) and 5-year progression-free survival for stage I patients (37% vs 17% [100]) (summarized in [102]). BCG is still nowadays the treatment of choice for bladder CIS. However, considering some tolerability issues, alternative immunotherapeutic approaches exploiting the immunogenic components of the mycobacterium (purified cell wall content, CpG DNA) have been trialed in preclinical and clinical studies (summarized in [103]). Almost 20 clinical trials (8 completed, 11 active/recruiting or not—www.clinicaltrials.gov) investigate strategies that could either enhance the efficacy of BCG or replace it with lower toxicity (Table 2).

The precise mechanism by which BCG exerts its antitumor effects is still not well understood. BCG effects do not seem to be mediated by direct proapoptotic effect but rather through a nonspecific immune response [104]. BCG indeed induced a massive increase in the proportion of T helper 1 (Th1) CD4 and γδ T cells, while no significant variation of CD8 and NK cells was found [105]. In vitro killing of human T24 bladder cancer cells induced by live BCG-infected DCs was mostly achieved by γδT cells and NKT cells but not conventional $CD8^+$ CTLs [106]. DC and macrophages are stimulated by BCG preparations through sensing of cell wall skeleton, peptidoglycan, and bacterial DNA by TLR2/4 [107] and TLR9 [108], respectively.

In therapeutic conditions (i.e., following chemotherapy), dead tumor cells express DAMPs that mediate the activation of a protective immune response through the triggering of innate sensors. The group of L. Zitvogel showed over the years that anthracyclin-induced cell death of tumor cells induces expression of cell surface (calreticulin [109]) and soluble molecules (HMGB1 [91], ATP [110]) resulting in tumor elimination by tumor-specific T cells (reviewed in detail in [111]). For instance, the release of HMGB1 by dying cells was shown to trigger TLR4 on DC [91]. Functional TLR4 was shown to be critical for anthracyclin-mediated antitumor response both in mouse model and in individuals with breast cancer carrying a loss-of-function allele of *TLR4* [91]. Those data were recently confirmed in vitro in human cellular models [112]. Interestingly, the activation of the NLRP3 inflammasome in DC is crucial as well for generation of tumor-specific T cell [110]. Those data suggest the possible need for the activation of multiple PRR pathways for efficient antitumoral activity.

Adjuvant activity of purified TLR ligands

Lipid A, the biologically active portion of LPS, is known to have potent immunostimulatory properties and has been evaluated for decades as an adjuvant for promoting immune responses to minimally immunogenic antigens, including tumor-associated antigens. Although several lipid A species have been tested, only 3-O-desacyl-4′-monophosphoryl lipid A (MPL) has been evaluated as a cancer vaccine adjuvant in published human clinical trials. MPL, which lacks the saccharide group and most phosphates present in LPS, induces many of the immunostimulatory properties of LPS but is at least 100-fold less toxic. Adjuvant System 04 (AS04, GlaxoSmithKline (GSK)), consisting of MPL and aluminum hydroxide, is currently a component in two licensed vaccines, Cervarix™ and Fendrix™, against human papillomavirus (HPV) and hepatitis B virus (HBV), respectively. AS04 is the first TLR ligand approved as a vaccine adjuvant in humans worldwide. The benefit of combining MPL to aluminum salt has been shown by better adaptive immune responses to immunization with HPV VLP vaccines [113]. MPL leads to a rapid activation of innate immune response that could explain the strong adaptive responses achieved with AS04-adjuvanted vaccines [114]. Clinical trials are being conducted to evaluate the safety and/or efficacy of cancer vaccines containing MPL combined with other immunostimulants, such as cell wall skeleton of *Mycobacterium phlei*, the saponin QS-21 or with QS-21 and CpG ODN [115] (Table 2).

GSK AS02B (MPL/QS21) or AS15 (CpG/MPL/QS21) adjuvant preparations were tested in melanoma-associated antigen 3 (MAGE-A3) protein vaccine in phase II clinical trials in patients with melanoma and non-small cell lung carcinoma (NSCLC). The adjuvant was shown to be essential for the development of strong humoral and cellular responses against MAGE-A3 epitopes [116]. Combination of MAGE-A3 and AS15 yielded higher specific immune responses and long-lasting clinical response [117] than with AS02B, which lack TLR9 agonist CpG. Large, double-blind phase III clinical trials testing MAGE-A3/AS15 combination (GSK1572932A) in patients with advanced melanoma or NSCLC are currently ongoing. Multicenter, open-label phase I/II trials are currently recruiting to test safety and efficacy of recombinant HER2 protein with AS15 adjuvant in patients with metastatic breast cancer.

MPL adjuvant is also a component of Stimuvax®, a lyophilized liposomal preparation containing BLP25 (MUC1 core peptide) lipopeptide. Phase IIB trial in patients with stage IIIB or IV NSCLC showed an increase in median survival time of 4.5 months in patients receiving BLP25 plus best supportive care (BSC) vs BSC alone [118]. Based on those data, phase III trials have been conducted in NSCLC and breast cancer. As a result of suspected

unexpected serious adverse reaction, the company has temporarily suspended those trials (press release).

Finally, DETOX™ adjuvant (Biomira, Inc.), combining MPL and *M. phlei* wall extract, was administered with cellular or peptide vaccines in phase I/II studies to patients with metastatic breast cancer, melanoma, colon, pancreatic, or lung cancer. A vaccine (Theratope™) combining synthetic carbohydrate molecules (sialyl-Tn (STn)), KLH, and DETOX™ was shown to induce strong anti-STn IgG and IgM responses with minimal side effects in metastatic breast cancer patients [119]. However, recent results from a phase III trial in a large cohort of metastatic breast cancer patients showed no overall benefit in time to progression or survival [120].

Besides its direct antitumoral effect, BCG has been evaluated as well with cellular vaccines in colorectal cancer and melanoma [121, 122]. Clinical benefit along with good safety profile was observed in those clinical trials, though insufficient control arms make it hard to conclude on the benefit of BCG on the actual regimen [121, 122]. Ongoing trials are testing BCG benefit in the adjuvant treatment of melanoma, neuroblastoma, and breast, lung, colon, and ovarian cancer (www.clinicaltrials.gov).

TLR3 ligands

TLR3: ligands and cellular expression

Synthetic Poly[I:C] and Poly[A:U] are known for decades for their potent inhibitor effects on the growth of virus-induced and virus-independent mouse tumors [123, 124]. However, it was only in 2001 that TLR3 was identified as a sensor of dsRNA by Alexopoulou et al. [80]. As previously emphasized, the effects of dsRNA on cancer must not necessarily be attributed to TLR3, as several cytosolic receptors exist [9], which may all contribute to tumor control. Nevertheless, several recent reports have established that tumor cells of various origins express TLR3—its expression can be upregulated by type I IFNs—and that its activation by dsRNA inhibits tumor cell survival. Besides exerting intrinsic activities on TLR3-expressing cancer cells, dsRNA activates the cells from both innate and adaptive immunity and modulates the tumor microenvironment. Those extrinsic roles of TLR3 on tumor progression reflect the expression of the receptor, present not only in immune cells but also in non-immune cells, including epithelial cells, endothelial cells, and fibroblasts.

Intrinsic roles of TLR3 in cancer cells growth, metastasis, and death

To date, the direct inhibition of tumor growth by TLR3 agonists has been reported in vitro for human breast, melanoma, prostate, head and neck, multiple myeloma, clear renal carcinoma, colon, lung, and cervical cancer cells [125–137], suggesting that targeting TLR3 could represent an opportunity for antitumor therapy. TLR3 activation inhibits the growth of cancer cells through two main processes: (a) decrease of proliferation and (b) induction of apoptotic cell death.

A decrease of cell proliferation in response to TLR3 activation by Poly[I:C] dsRNA has been demonstrated by BrDu incorporation experiments for breast and prostate cancer cells [130, 131] and likely participates to the dsRNA antitumoral effect in the other types of cancers listed above. The molecular mechanism remains so far uncertain but could be related to autocrine IFN signaling and blockade of cell cycle through combined downregulation of cyclin D1 and upregulation of cyclin-dependent kinase inhibitor p27 [44, 130, 131]. In contrast, Poly[I:C] stimulation has been reported to increase cell proliferation of head and neck and multiple myeloma cell lines in a c-Myc- and NF κB-dependent manner, respectively [137, 138]. Although TLR3 requirement for these effects remains to be clearly established, production of IL-6 vs IFNα by multiple myeloma cells in response to Poly[I:C] could switch cell decision to proliferate or die [137].

The first demonstration of a direct antitumoral effect of TLR3 activation on tumor cells was reported for breast cancer cell lines in which Poly[I:C] treatment induces caspase-8 activation and caspase-dependent apoptotic cell death [130]. Requirement for caspase-8 activation in TLR3-induced apoptosis was further confirmed in other cancers such as melanoma, nasopharyngeal, prostate, and cervical cancers [127, 129, 131, 134–136] indicating that the extrinsic pathway of apoptosis is triggered by TLR3. TLR3 death-signaling pathway is still under intensive investigations. A recent report [136], as well as our submitted data, led to the identification of a proapoptotic molecular complex containing caspase-8 and responsible for TLR3-induced cell death in cancer cells. The molecular basis of this death complex reminds that of classical death receptors of the TNFR superfamily such as TNF or TRAIL receptors [139]. Its formation requires the protein RIP1, a kinase playing a key role in the integration of signaling pathways from genotoxic stress, death receptors, or microbial infections and closely involved in the cell's decision to live or die [140].

Triggering the extrinsic pathway of apoptosis by TLR3 activation in cancer cells leads the way to combine TLR3 ligands with conventional therapies, such as chemotherapies or radiotherapy that preferentially target the intrinsic mitochondrial pathway of apoptosis, to improve clinical outcomes. In vitro combinations of Poly[I:C] with 5-fluorouracil, cisplatin, or etoposide anticancer drugs indeed increase cell death of HCT116 colon cancer cell line[128]. These in vitro preliminary data are encouraging but need to be extended to other drugs and cancers and to be validated with in vivo models. Furthermore,

detailed understanding of molecular mechanisms of TLR3-induced apoptosis in cancer cells should help to the design of novel sensitizing combinations. Typically, Smac mimetic compounds (currently in phase I clinical trial), which inhibit the inhibitor of apoptosis proteins (IAPs) XIAP, cIAP1, and cIAP2, strongly synergize with dsRNA for inducing the apoptosis of various tumor cell lines [127, 134–136]. The molecular mechanism may rely on alterations of RIP1 ubiquitination and stability (our unpublished data). Recently, a large signaling platform termed Ripoptosome has been described to transmit apoptotic and necrotic signals emanating from TLR3 [136]. In response to Smac mimetic compounds, this molecular complex assembles spontaneously in some tumor cells, a feature that could be linked to the level of stress signals present in cancer cells compared to normal cells [136, 141]. The absence of Ripoptosome might explain why normal cells are not killed by Poly[I:C] alone or in combination with IAP antagonists, but undergo only a moderate proliferation slowdown ([134] and our unpublished data). Thus, the benefits of combining IAP inhibitors with TLR3 ligand should be further investigated, notably in preclinical animal models.

Interestingly, phase II clinical trials on glioblastoma suggest that poly-ICLC (Poly[I:C] stabilized with polylysine and carboxymethylcellulose) may improve patient survival when combined with radiotherapy alone or with radiotherapy plus the alkylating agent temolozomide [142, 143] (Table 3). Current phase II trial is analyzing intramuscular injection of poly-ICLC for the treatment of low-grade gliomas. The relevance of TLR3 expression in cancer cells for dsRNA antitumor effects has been recently demonstrated in several immunodeficient mouse models [59]. Moreover, retrospective analysis of primary breast cancers by the use of a new monoclonal antibody anti-TLR3 revealed that TLR3 expression by the tumor is a biomarker for the therapeutic efficacy of dsRNA on metastatic relapse [59]. Complementary data have been obtained with syngeneic tumor mouse models expressing TLR3, for which Poly[A:U] exerts its antitumoral effects by acting on both host and tumor cells TLR3 [144]. While confirming the potential of TLR3 stimulation in immunotherapy (see below), and stressing a role of this receptor in tumor immune surveillance, another study using syngeneic and transgenic mouse prostate tumor concluded that the antitumoral effect of dsRNA was mainly dependent on TLR3-expressing host cells [145]. Such discrepancies might reflect key differences between human and mouse TLR3, regarding both the expression and the signaling of apoptosis and of cytokine induction [146–148]. Indeed, most mouse tumor cells lines were found to be resistant to dsRNA/TLR3-induced apoptosis compared with human tumors (unpublished data). Such variances between mouse and human cancer cells must be kept in mind when transposing to human the results of dsRNA/TLR3-based immunotherapy assays carried on in mouse.

Extrinsic roles of TLR3-L on tumor environment and adjuvant activities

The capacity of TLR3 ligands to activate mDC appears to be the major determinant of its adjuvant ability. Several phase 0/I/II clinical trials are in progress to determine the potential of dsRNA poly-ICLC as adjuvant for antigen peptide vaccinations for various types of cancer (Table 3). Ampligen® Poly[I:C(12)U] (Hemispherx Biopharma of Philadelphia), a GMP-grade synthetic analog of Poly(I:C) shown to specifically target TLR3 [149] is under investigation as an anticancer drug [150]. Poly[I:C(12)U] was previously tested in the treatment of chronic fatigue and AIDS and is currently under clinical investigation with tumor cell lysate for the treatment of ovarian, fallopian, or peritoneal cancers.

Poly[I:C] triggers the upregulation of co-stimulatory molecules on DCs that is partially TRIF-dependent [151] and increases the cross-presentation of exogenous antigen to $CD8^+$ T cells [152]. Combined with antigen, TLR3 agonists thus drive a potent and type I IFN-dependent antigen-specific $CD4^+$ Th1 response [153] and IFN γ production by $CD8^+$ T cells [154].

Secretion of type I IFN and IL 12 by DC or by other accessory cells in response to Poly[I:C] is also responsible for the indirect activation of NK cells associated with increased cytotoxicity and IFN γ secretion [155, 156]. In addition, the membrane-associated tetraspanin-like named IRF 3-dependent NK-activating molecule was shown to be upregulated on the surface of mDC by Poly[I:C] stimulation and to participate to the activation of NK cells via cell–cell contact. Besides these soluble and membrane-bound signals provided by DC, NK requires in addition a direct stimulation of RIG-I by dsRNA to become activated [156].

Related to those combined effects on T cells and NK cells, TLR3 activation was shown to be critically important for cross-presentation of viral antigen by DCs [157] and for priming antiviral $CD8^+$ T cells during acute viral infection. Moreover, Poly[I:C] has been shown to be among the most powerful adjuvant for tumor vaccine [158, 159] requiring both TLR3 [160] and MDA5 [153, 161] to obtain fully protective and long-term immunization. The adjuvant properties of Poly[I:C] in tumor vaccine can still be increased by preventing the type I interferon-dependent myeloid DC apoptosis [154] and the upregulation of B7-H1 co-inhibitory molecule expression by DC [162] that both limit the magnitude of the CD8 T cell response

TLR3 activation triggers the secretion of various chemokines by cells of the microenvironment. Through these chemokines, macrophages, neutrophils, and lymphocytes can be recruited in situ and participate in wound healing [163] or, in the case of cancer, either to the eradication of the tumor or to the establishment of local immunosuppression. Indeed, uncoupling the effect of the immunosuppressive

Table 3 TLR3 ligand in human clinical trials

Effect[a]	Clinical phase	Status[b]	Designation[c]	Cancer type	Reference
A	Phase 0	R-2009	s.c. Poly-ICLC adjuvant for antigen–peptide vaccination	Gliomas	NCT00874861
A	Phase 0	R-2010	Poly-ICLC adjuvant for antigen–peptide vaccination	Pediatric gliomas	NCT01130077
A	Phase 0	R-2008	Poly-ICLC adjuvant for PSMA and TARP peptide vaccination	Prostate cancer	NCT00694551
A	Phase 0	R-2008	s.c. Poly-ICLC adjuvant for antigen–peptide vaccination	Astrocytomas and oligoastrocytomas	NCT00795457
A	Phase 0	R-2009	i.m. Poly-ICLC adjuvant for MUC1 protein vaccination	Triple-negative breast cancer	NCT00986609
A	Phase I	A/R	s.c. Poly-ICLC adjuvant for NY-ESO-1 OLP4 protein vaccination	Epithelial ovarian, fallopian tube, and primary peritoneal cancer	NCT00616941
A	Phase I	R-2009	s.c. Poly-ICLC adjuvant for NY-ESO-1/gp100/MART-1 peptide vaccination	Melanoma	NCT01008527
A	Phase I	S	Poly-ICLC adjuvant for MUC1 protein vaccination	Prostate cancer	NCT00374049
A	Phase I/II	R-2010	s.c. Poly-ICLC adjuvant for NY-ESO-1 protein vaccination	Melanoma	NCT01079741
A	Phase I/II	R-2011	s.c. Ampligen adjuvant for oxidized tumor cell lysate vaccination	Ovarian, fallopian tube, or primary peritoneal cancer	NCT01312389
A	Phase I/II	R-2011	Ampligen adjuvant for HER2 protein vaccination	HER2-positive breast cancer	NCT01355393
A	PhaseII	R-2008	s.c. Poly-ICLC adjuvant for MUC1 peptide vaccination	Colorectal cancer	NCT00773097
D	Phase I	C-2005	NS-9 (Poly I: Poly C)	Liver metastases from various primary cancers	NCT00094003
D	Phase I	T	Poly-ICLC + radiation	Low-grade recurrent B and T cell lymphoma	NCT00880867
D	Phase II	C-2009	poly-ICLC + radiation	Brain and central nervous system tumors	NCT00052715
D	Phase II	R-/2010	Intramuscular Poly-ICLC	Low-grade B gliomas	NCT01188096

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[a] Effect: *A* adjuvant, *D* direct; effect of therapeutic combination

[b] Clinical trial status: *A* active, *R* recruiting, *C* completed, *W* withdrawn, *T* terminated, *S* suspended; with completed date or starting date for active and recruiting trials

[c] Clinical trial designation: *s.c.* subcutaneous, *i.m.* intramuscular

chemokine CCL5 from the release of the immunostimulatory CXCL10 has been shown to augment the anticancer efficacy of TLR3 ligands [144]. Moreover, injection of Poly [I:C] was found to increase the infiltration of T lymphocytes and NK cells within mouse prostate tumor and to enhance tumor eradication [145]. Lastly, synthetic dsRNAs may also interfere in different ways with tumor vascularization by stimulating tumor cells to secrete VEGF [164], by directly increasing the pro-coagulant functions of the endothelial cells that express TLR3 [165], or indirectly by driving the secretion of IFN-γ by immune cells present at the site of the tumor [166].

In conclusion, TLR3 agonists represent promising components of new drug combinations for cancer therapy due to the synergy of direct inhibitory effects on TLR3-expressing cancer cells and indirect activities on the immune and non-immune cells of the tumor microenvironment. Better understanding of the mechanisms of action of TLR3 and of the involvement of other receptors in the response to TLR3 agonists is now required for optimizing the therapeutic potential.

TLR7/8 ligands

TLR7/8/: ligands and cellular expression

TLR7 and TLR8 are structurally closely related members of the TLR family involved in virus recognition. hTLR7 is predominantly expressed in lung, placenta, and spleen. hTLR8 is more abundant in lung and peripheral blood leukocytes [167]. hTLR7 is expressed in B cells, pDC, and eosinophils and hTLR8 in monocytes and mDC including

recently described hBDCA3+ blood DC [8, 168]. In line with those data, hBDCA3+ DC responds to TLR8-L but not TLR7L [169, 170]. NK cells functionally express both TLR7 and TLR8 [171].

Viral single-stranded RNAs are the natural ligands for TLR7 and TLR8 [172, 173]. Even though redundant system of ssRNA recognition exists, mTLR7 was shown to be central for in vivo IFNα induction, mediated by pDC, in response to ssRNA viruses (VSV, influenza) [173]. Stimulation of TLR7 and TLR8 is sequence dependent. AU-rich sequences only stimulate hTLR8 responses [174]. GU-rich sequences can trigger the activation of human and mouse TLR7/8 with the exception of mTLR8 [172] leading to the belief that TLR8 is biologically inactive in mice. Recent analysis of TLR8-deficient mice showed increased TLR7 expression and subsequent response to its ligand and elevated serum levels of autoantibodies [175]. This suggests a central role for mTLR8 in the regulation of mTLR7 expression and prevention of spontaneous autoimmunity. However, this highlights a major difference between human and mice in term of response to TLR8-L. Results from preclinical studies in mouse should thus be taken very carefully for transfer to clinical trials.

Additionally, Jurk et al. recently identified a non-uridine-rich RNA sequence motif on a phosphodiester backbone that induces strong IFNα production by PBMC through TLR7 [176]. Silencing RNAs (siRNAs) also trigger the production of IFNα and IFNγ in vivo and in vitro [177, 178]. Their immunostimulatory potential is mediated by PRRs, including TLR7/8 [177, 178] but also TLR3 [179] and RIG-I [71]. Although RNA-based compounds would appear to be interesting candidates for therapeutic approaches, susceptibility to nuclease degradation limits their use. New class of synthetic TLR7/8 agonists referred to as stabilized immune-modulatory RNA compounds have been developed [180, 181]. They display increased stability, induce cytokine/chemokine production in vitro, and were shown to trigger potent antitumor activity in colon and lung cancer model [180]. This antitumoral activity was associated to a decrease in $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) and increase TA-specific IFNγ response [182]. Finally, synthetic small molecules such as imidazoquinolines and nucleoside analogs also activate TLR7 and/or TLR8. Imiquimod® (R837), Gardiquimod, CL097 (3M-001), and Loxoribine preferentially activate TLR7 in contrast to CL075 (3M-002) that mainly targets TLR8. Resiquimod® (R848) triggers both receptors and was shown to be more soluble and more potent in inducing cytokine expression than its family member imiquimod.

Antitumoral efficacy of TLR7/8 agonists as monotherapy in human trials

Two topical formulations of Imiquimod, Zyclara™ (Graceway Pharmaceuticals; 3.75% imiquimod) and Aldara™ (Graceway Pharmaceuticals; 5% imiquimod), have received legal approval in 1997 for the treatment of HPV-related warts and skin keratosis. Aldara™ is approved as well for the treatment of small superficial basal cell carcinoma (BCC) by the FDA and the European Union. Combined clinical and histological assessments showed a 73–75% clearance rate of BCC in two phase III clinical trials [183]. Imiquimod is currently in phases III (with cryosurgery) and IV (with IFNα) trials in patients with BCC. Besides BCC, objective responses have been observed in some patients with melanoma or lentigo maligna (melanoma in situ) when imiquimod was applied to skin metastasis [184–188]. Finally, two ongoing trials are assessing imiquimod topical treatment in patients with breast cancer with chest wall recurrence or skin metastasis (phase II).

More recently, a specific TLR7 agonist, 852A (3M Pharmaceuticals), is being tested in patients with solid tumors and hematologic malignancies. Unlike imiquimod, 852A has pharmaceutical properties that enable it to be formulated for systemic delivery. Initial data from phase I trial showed that intravenous injection of 852A was well tolerated and induced systemic immune activation [189, 190] that eventually resulted in prolonged disease stabilization in patients with stage IV metastatic melanoma [190]. 852A delays tumor growth in mouse model via inhibition of tumor cell line proliferation through type I IFN induction by pDC [191]. 852A is currently in phase II clinical studies for treatment of cancer, including melanoma. Finally, VTX-2337 (VentiRx Pharmaceuticals), a new specific TLR8 ligand, is in phase I/II clinical trial in combination with current regimen for the treatment of lymphoma and various solid tumors. Results of phase I trial in advanced solid tumors including colorectal (n=9), pancreatic (n=6), and melanoma (n=5) cancers showed good safety and tolerability [192].

Topical application of such TLR7/8 agonists is highly effective, whereas their systemic application has been largely unsuccessful for cancer treatment. Explanations might be variable in tumor type and stages, tumor accessibility, as well as the induction of immune unresponsiveness upon systemic injection of TLR ligands. To circumvent such state, Bourquin et al. designed a novel protocol of treatment with repeated R848 injections and treatment-free intervals and showed improvement of the efficacy of cancer therapy [193]. Treatment failure in solid tumors has been associated in a mouse breast cancer model to increased levels of IL 10 [194]. Blockade of IL 10, but not TGFβ, enhanced the antitumor effect of imiquimod by significantly improving the survival in treated mice [194].

Strong cellular antitumor immune responses have been observed in skin tumors treated topically with dual TLR7/8 ligands. pDCs have been involved in antitumoral immunity, especially upon imiquimod treatment. Topical imiquimod

application resulted in pDC recruitment to skin lesions in patients with BCC. Clinical response to imiquimod treatment was correlated to pDC number in a melanoma mouse model [195]. Antitumoral activity of pDC was correlated to tumor cell apoptosis via type I IFN production [168] and TRAIL upregulation [196]. In addition to TLR7-induced IFNα production by pDC, TLR8 triggering on human monocytes and mDC leads to the secretion of proinflammatory cytokines such as TNFα, IL12, and MIP1 [197]. The antitumoral activities of TLR7/8 agonists has thus been associated to their ability to induce bioactive IL12p70 secretion by DC, enhancement of NK cell activation [198], as well as a decrease in $CD4^{+}CD25^{+}Foxp3^{+}$ Treg [199]. TLR8 ligand may even prove superior NK cell activation than imiquimod [198]. Their ability to induce a potent Th1-type immune response and decrease the number of Tregs could lead to a tumor microenvironment more favorable for the host immune system to respond strongly against the tumor.

While TLR7/8 agonists are potent immunomodulators, direct intrinsic effect on tumor cells shall be considered. A recent study indeed stressed the potential adverse effects of TLR7/8 agonists in the context of lung cancer [200]. TLR7/8 are naturally expressed in human lung epithelial cells and upregulated in lung tumor cells. Activation of those receptors was associated with increased tumor cell survival and resistance to apoptosis induced by several chemotherapies in vitro through NF-κB activation and *Bcl2* upregulation [200].

Adjuvant activities of TLR7/8 agonists

TLR7/8 ligands were shown to display as well strong adjuvant activities. TLR7/8 ligands (CL075 or R848) induced maturation of monocyte-differentiated DC. Moreover combination with [poly(I:C)] [201] or LPS [202] yielded DC that showed even better profiles in terms of phenotype, migration, as well as capacity to activate NK cells and IFNγ-producing $CD8^{+}$ CTL [201, 203]. Imiquimod is thus often associated to DC vaccines. Topical application of imiquimod in phase I trial was shown to augment the immunogenicity of melanoma peptide vaccine when administered with systemic FLT3L, to mobilize DC [204]. Those data suggested a potent effect of imiquimod to activate in situ DC to uptake the antigen and induce specific T cell response. Follow-up trial of topical resiquimod in combination with a NY-ESO1 protein in montanide emulsion is ongoing. Several trials are currently testing the vaccination of patients with tumor lysate-loaded DC in the context of brain tumors (phase II) (Table 4). Imiquimod and gardiquimod, two TLR7 ligands, were indeed shown to improve the antitumor effects of tumor lysate-loaded DCs in murine models [205, 206]. Vaccination in patients with ovarian cancer with dendritic cell/tumor fusions with granulocyte–macrophage colony-stimulating factor (GM-CSF) and imiquimod is also being tested (phase II). Finally, Celldex Therapeutics is testing its antibody-based combination immunotherapy in phase II trials in bladder cancer (CDX-1307 co-administered with GM-CSF, Poly-ICLC, and Resiquimod) and various NY-ESO1 malignancies (CX-1401 along with Resiquimod and/or Poly-ICLC). This technology allows targeting of peptide vaccine to DC using antibodies that specifically target those cells (anti-DEC205, anti MR) inducing antigen uptake, further cross-presentation to specific T cells to induce protective antitumoral immune response as shown in mouse models [207, 208]. Targeting NY-ESO-1 to DC using CDX-1401 with topical resiquimod induces robust and broad humoral and cellular responses in advanced cancer patients (press release). CDX-1307 trial has been terminated for unknown reasons (http://clinicaltrial.gov).

TLR7/8 ligands are also used as adjuvants in protein and peptide vaccines in clinical trial in melanoma [MAGE-3 (two phases I/II), gp100 (two phase II), NY-ESO-1 (phase I completed [209], phase I)], glioma (phase I), and neuroblastoma (phase I) (Table 4). Published results from first phase I trial described the use of imiquimod in a series of vaccinations against the NY-ESO-1 antigen in patients with malignant melanoma [209]. Topical imiquimod is well tolerated and induces local inflammation with a high recruitment of APC and T cell. Overall, the vaccine combination induces measurable NY-ESO-1-specific antibodies and IFNγ + CD4 + T cell responses [209].

TLR9 ligands

TLR9: ligands and cellular expression

TLR9 expression is limited in tissue distribution. In humans, its constitutive expression is confined to B cells, pDCs, and polymorphonuclear leukocytes [32–34]. TLR9 is similarly expressed on mouse B cells, macrophages, and neutrophils; however, major differences were observed in mouse DC as all DC subpopulations do express TLR9 [210]. Moreover, TLR9 has been reported to be expressed by some non-immune cells such as gut, respiratory and cervical epithelia [211, 212], and keratinocytes [44, 213].

TLR9 activity has been characterized mainly in response to the synthetic CpG motif-containing ODN [214]. These motifs are expressed as unmethylated CpG motifs in bacterial or viral DNA. It has been shown that TLR9 is critical in the control of bacterial infections with *Brucella abortus* [215], *Streptococcus pneumoniae* [216], and *Mycobacterium tuberculosis* [217]. TLR9 polymorphisms have been associated with an increased risk for *Helicobacter* infection, which has high incidence in gastric cancers, suggesting that TLR9 is involved in recognition and clearance of *Helicobacter* [218]. TLR9 also detects many DNA viruses including HSV type 1 and type 2 [219, 220], murine cytomegalovirus [220, 221], adenovirus

Table 4 TLR7/8 ligand in phase II/III/IV human clinical trials

Effect[a]	Clinical phase	Status[b]	Designation	Cancer type	Reference
A	Phase I/II	A/R	Daclizumab	Brain and central nervous system tumors	NCT00626483
A	Phase I/II	A/R	IMA910 plus GM-CSF with cyclophosphamide	Colorectal carcinoma	NCT00785122
A	Phase I/II	R-2010	Peptide vaccination associated with vaccine MAGE-3.A1 peptide, or the NA17.A2 peptide + IL-2, IFN-α and GM-CSF, imiquimod	Metastatic melanoma	NCT01191034
A	Phase I/II	R-2009	CDX-1401 with adjuvant	Solid tumors	NCT00948961
A	Phase II	C-2006	Adjuvants accompanying peptide immunization (gp100)	Melanoma	NCT00273910
A	Phase II	C-2010	CYT004-MelQbG10 vaccine with and without adjuvant	Malignant melanoma	NCT00651703
A	Phase II	R-2010	Tumor vaccine, gp100, with TLR agonist	Melanoma	NCT00960752
A	Phase II	R-2008	Vaccination with DC/tumor fusions with GMCSF and imiquimod	Ovarian cancer	NCT00799110
A	Phase II	R-2010	Topical resiquimod adjuvant for autologous tumor lysate pulsed DC vaccination	Brain tumors	NCT01204684
A	Phase II	R-2010	Dendritic cell vaccine for patients with brain tumors	Brain tumors	NCT01204684
A	Phase II	R	Vaccination with DC loaded with brain tumor stem cells	Brain tumors	NCT01171469
A	Phase II	R-2010	Topical resiquimod adjuvant for MAGE-3/gp100 protein vaccination	Melanoma	NCT00960752
A	Phase II	T	CDX-1307 vaccine regimen in patients	Bladder cancer	NCT01094496
D	Phase I/II	A	VTX-2337 + radiotherapy	Low-grade B cell lymphoma	NCT01289210
D	Phase I/II	R-2011	VTX-2337 + local radiation	Low-grade B cell lymphoma	NCT01396018
D	Phase II	A/R	Topical imiquimod	Breast cancer	NCT00899574
D	Phase II	C-2006	852A	Mestatic cutaneous melanoma	NCT00189332
D	Phase II	C-2008	852A	Breast, ovarian, endometrial, and cervical cancers	NCT00319748
D	Phase II	R-2008	Topical imiquimod and Abraxane	Breast cancer cutaneous metastases	NCT00821964
D	Phase II	T	852A	Hematologic malignancies	NCT00276159
D	Phase II/III	R-2011	Imiquimod	Lentigo maligna	NCT01088737
D	Phase III	A/R-2002	Topical imiquimod compared with surgery	Basal cell carcinoma	NCT00066872
D	Phase III	C-2007	Topical imiquimod	Basal cell carcinoma	NCT00189241
D	Phase III	C-2007	Topical imiquimod basal cell carcinoma	Basal cell carcinoma	NCT00189306
D	Phase III	C-2010	Cryosurgery and imiquimod	Basal cell carcinoma	NCT01212549
D	Phase III	C-2007	Topical imiquimod	Basal cell carcinoma	NCT00189280
D	Phase III	R-2007	Topical imiquimod and cryosurgery	Basal cell carcinoma	NCT01212562
D	Phase IV	C-2005	Topical imiquimod aftern curretage	Basal cell carcinoma	NCT00314756
D	Phase IV	C-2005	Topical imiquimod	Basal cell carcinoma	NCT00204555
D	Phase IV	R-2010	Topical imiquimod	Lentigo maligna	NCT01161888
D	Phase IV	R-2011	Intron-A/Aldara combination therapy	Basal cell carcinoma	NCT00581425

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[a] Effect: *A* adjuvant, *D* direct; effect of therapeutic combination

[b] Clinical trial status: *A* active, *R* recruiting, *C* completed, *W* withdrawn, *T* terminated, *S* suspended; with completed date or starting date for active and recruiting trials

[220, 222], HPV CpG motifs [57], epstein barr virus (EBV) [223], and recently, adenovirus-associated virus [224]. In addition to the CpG motifs, recent studies have indicated an important role of nucleotide derivatives and the DNA sugar backbone in determining TLR9-dependent immune stimulatory activities [225]. There is increasing evidences suggesting that TLR9 can also sense DAMPs. The release of DAMPs can be linked to the ability of TLRs to induce an immune response

and/or to control mechanisms that control tissue integrity [226–229]. For instance, pDCs were shown to sense genomic DNA associated to the cationic antimicrobial peptide LL37. Such complex triggers robust IFN responses by activating endosomal TLR9 thus leading to robust Th1-mediated adaptive response [230]. TLR9 activation by self-DNA–LL-37 conjugate was shown to be involved in autoimmune diseases, as psoriasis and systemic lupus erythematosus [230].

TLR9 recognizes CpG motifs as well as self-DNA itself with certain structures. There are three structurally distinct classes of CpG ODN: CpG-A, CpG-B, and CpG-C. CpG-A preferentially stimulates pDCs to produce type I IFNs, but very little maturation of APC and B cell activation [231]. CpG-B induces very strong B cell proliferation and differentiation and APC maturation and low levels of type I IFN production. CpG-C has combined activities of CpG-A and CpG-B [231]. The optimal CpG motif in mice consists of an unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines, whereas the optimal motif in humans is TCGTT and/or TCGTA [232].

Two important events for TLR9 signaling were unraveled in the recent years using mouse models. First, localization of TLR9, as TLR3 and TLR7, to the endosome is crucial for its function and requires the multi-transmembrane protein domain-containing protein named UNC93B [233–235]. Second, proteolytic cleavage in an endolysosomal compartment is required for TLR9 activation by CpG [236–238]. In addition, TLR7 and TLR3 were recently shown to be processed in an analogous manner [239].

pDC and B cell activation by TLR9 in response to CpG motifs lead to a robust inflammatory and type I IFN immune responses. IFNα secretion will in turn modulate DC biology by increasing their APC function and migratory capacities, as well as B and T cell trafficking to the lymph node [240]. Ligand activity on TLR9 expressed in human B cells induces proliferation, class-switch recombination, and enhanced antigen-specific antibody production [241]. TLR9 agonists on human B cells induce the expression of the cytokine and chemokine genes (IL-6, MIP1-α, MIP1-β, TNF-α…) as well as the co-stimulatory molecules, Fc receptors, but also anti-apoptotic gene (*bcl2*), the transcription factors MYC and TCFL5, and genes critical for B cell proliferation and differentiation [242]. TLR9 activation, along with B cell antigen receptor, induces naive B cell differentiation into plasma cells. Direct conjugation of antigen and CpG reveals a mechanism that may operate during the initiation of primary immune responses, leading to enhanced antigen-specific B cell proliferation and differentiation to form extrafollicular plasma cells [243, 244].

TLR9L as immunotherapy in cancer treatment

TLR9 CpG ODNs have been added to the large list of anticancer drugs as monotherapy or in combination with chemotherapy, radiotherapy, and other immunotherapeutic approaches as they increase antigen presentation and boost antitumor T and B cell responses. Several synthetic TLR9 agonists have been developed for clinical grade use and displayed substantial efficacy in the preclinical and clinical models (discussed in detail in [245]). Sequences dedicated to stimulating TLR9 are termed immune-modulatory ODN (IMOs). IMO-2055 (Idera Pharmaceuticals/Merck KGaA) was described in a mouse model when used as a monotherapy and its antitumoral activity was amplified when it was used in combination with chemotherapeutic agents [246, 247] (Table 5). This compound is currently being analyzed in two phase Ib trials in NSCLC (with bevacizumab and erlotinib) and colorectal cancer (with cetuximab and irinotecan) [248]. Based on increased incidence of neutropenia and electrolyte imbalances reported in its phase 1 trial in patients with squamous cell carcinoma of head and neck (SCCHN), Merck will not conduct further clinical development of IMO-2055 at this stage (press release July 2011).

A second panel of ODN has been created termed immunostimulatory sequences (ISS). These short DNA sequences increase the activity of TLR9 and enhance the production of Th1 cells. ISS can be linked to antigens or used alone to suppress a Th2 response. The lead compound ISS-1018 (Dynavax) induces the production of immunoglobulin and type I IFN in vitro by B cells and of IFNβ, IL-12, and TNF-α by pDC [249]. Potential enhancement of antibody-dependent cell-mediated cytotoxicity is also regarded with interest, and studies are ongoing with ISS-1018 in combination with the anti-CD20 or anti-HER2 in lymphomas and breast cancer patients, respectively [250]. The ISS-1018 ODN sequence has been also used in clinical trials alone and combined with current regimen for non-Hodgkin's lymphoma and metastatic colorectal cancer. CpG-7909 (also known as Agatolimod or PF3512676, Pfizer) is another CpG-B-type ODN that is being developed in therapeutic combinations to treat cancers as well as allergy, asthma, and HBV infection (Table 5). The use of CpG-7909 is discussed in depth in the review by Holtick et al. [245]. It is currently in phase II clinical trials for treatment of melanoma, cutaneous T cell, non-Hodgkin, and follicle center and marginal zone B cell lymphomas. However, evaluation in phase III trials in NSCLC was discontinued when it did not show increased efficacy over standard chemotherapy [251]. Interesting phase I/II results have recently been presented by Brody and colleagues. In this study, the combination of local radiotherapy and CpG-7909 to lymphoma sites led to tumor regression even at distant tumor localizations [252].

CpG ODN as adjuvant in peptide vaccine trials

MGN-1703 and MGN-1706 (Mologen AG) are double stem-loop immunomodulating adjuvants that consist of noncoding DNA and are being developed as anticancer TLR9 agonists (Table 5). Individually, these compounds have anticancer

Table 5 TLR9 ligand in human clinical trials

Effect[a]	Clinical phase	Status[b]	Designation	Cancer type	Reference
A	Phase I	C	NY-ESO-1 protein combined with CpG 7909 and montanide	Various tumors	NCT00299728, NCT00199836
A	Phase I	C-2006	NY-ESO-1 protein combined with CpG 7909	Prostate cancer	NCT00292045
A	Phase I	T-2010	CpG with autologous KLH/TT vaccine	Lymphoma, various plasma cell neoplasma	NCT00369291
A	Phase I	U	gp100/MART1 vaccine + CpG7909 + GMCSF	Stage III or Stage IV melanoma	NCT00471471
A	Phase I	U	Autologous tumor cell-TLR9 agonist	Colorectal cancer	NCT00780988
A	PhaseI	R-2005	CPG + montanide + tumor antigen vaccine	HLA-A2+ Stage III/IV melanoma	NCT00112229
A	PhaseI/II	C- 2003	CPG7909 with herceptin	Breast cancer	NCT00043394, NCT00031278
A	Phase I/II	T-2007	Cp7909 + MAGE-3	Melanoma	NCT00145145
A	Phase II	C	gp100/MAGE6A3 vaccine, montanide, and CpG 7909	Melanoma	NCT00085189
A	Phase II	R-2006	URLC10-177 and TTK-567 peptide vaccine combined with CpG7909	Esophageal cancer vaccine	NCT00669292
A	Phase II	R-2009	CpG-activated whole cell vaccine followed by autologous immunotransplant	Mantle cell lymphoma	NCT00490529
D	Phase II	R-2011	IL2 and MAGE-3 vaccine with AS15 adjuvant	Melanoma	NCT01266603
D	PhaseII	R-2004	CPG + melanoma antigen peptide vaccines [Melan-A/Mart-1 (both EAA and ELA), NY-ESO-1b analog]	Stage III/IV melanoma patients	NCT00112242
D	PhaseII	R-2009	CPG7909 with trastuzumab	Breast cancer	NCT00824733
D	Phase I	A-2004	CpG7909	Chronic lymphocytic leukemia	NCT00233506
D	PhaseI	A-2008	IMO-2055 vs cetuximab or FOLFIRI	Colorectal cancer	NCT00719199
D	Phase I	C-2007	Cp7909	Breast neoplasms	NCT00043368
D	Phase I	C-2011	IMO-2055+ erlotinib + bevacizumab	Non-small lung carcinomas	NCT00633529
D	Phase I	R/2010	MGN-1703	Colorectal cancer	NCT01208194
D	Phase I	U	ISS1018 + irinotecan + cetuximab	Colorectal neoplasm	NCT00403052
D	Phase I/II	A-2004	CpG7909 with rituximab and radiation therapy	Lymphoma	NCT00438880
D	Phase I/II	C-2004	CpG7909	Renal cancer	NCT00043407
D	Phase I/II	C-2010	CpG7909 + radiation therapy	Lymphoma	NCT00185965
D	Phase I/II	C-2007	CPG 7909	T cell lymphoma	NCT00043420
D	Phase I/II	U	CPG 7909	T cell lymphoma	NCT00091208
D	Phase II	C	Cp7909	Melanoma	NCT00043368, NCT00070642
D	Phase II	C-2007	CpG with chemotherapy	Lung cancer	NCT00070629
D	Phase II	C-2008	IMO-2055	Renal cell carcinoma	NCT00729053
D	Phase II	C-2009	ISS1018 vs rituxan	Non-Hodgkin's B lymphoma	NCT00251394

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[a] Effect: *A* adjuvant, *D* direct; effect of therapeutic combination

[b] Clinical trial status: *A* active, *R* recruiting, *C* completed, *W* withdrawn, *T* terminated, *S* suspended; with completed date or starting date for active and recruiting trials

effects in preclinical models [253] and positive results were obtained in a phase I clinical trial of MGN-1703 in prostate and colorectal cancer. CpG7909 is being tested too as adjuvant in phase I/II clinical trials along with peptide vaccine, in esophageal cancer (URCL10-177 and TTK-567 peptides), NY-ESO-1 expressing tumors such as prostate cancer (with NY-ESO-1 antigen), and melanoma (MART1, gp100, MAGE-A3, Melan-A, MAGE10). CpG 7909 was tested with KLH and TT protein vaccine in treating patients who have undergone autologous stem cell transplant in phase I trial that

was terminated, as risk to benefit profile did not justify continuation of the trials. CpG7909 is being tested along with MPL-based AS15 adjuvant and IL-2 in recombinant MAGE-A3 peptide vaccine. Finally, CpGs are, as previously mentioned, a component of the AS15 adjuvant currently in phase III trials testing MAGE-A3 vaccine in patients with melanoma or NSCLC, and a phase II trial with rHER2 in patients with metastatic breast cancer.

Intrinsic activities of TLR9 in cancers

The role for TLRs in response to infection is clear, in that TLR9 recognizes PAMPs and responds by eliciting a robust antiviral response. However, viruses associated to cancers have developed clever strategies to transform the host cell of infection and also escape immune attack by deregulating somehow the immune response.

TLR9 is differentially expressed on tumor and healthy tissues. In some tumors, TLR9 has been found upregulated when compared to healthy tissue. In prostate or lung cancers, TLR9 and ERα expression is simultaneously increased especially in poorly differentiated tumors [254]. Conversely, a study on the clinical relevance of TLRs in breast cancer showed that tumors with high TLR9 expression by fibroblast-like cells were associated with low probability of metastasis [255]. On the opposite, TLR9 levels are abolished for instance during the transformation of bone marrow cells from myelodysplastic syndromes to overt leukemia [256] and in cervical cancers [57]. We found that TLR9 expression and function were decreased in cervical cancer cell lines and biopsies associated with high-risk type HPV but not with low-risk type HPV [57]. This downmodulation of *tlr9* was shown to be mediated by HPV16 oncoproteins via the Nκ-FB pathway [57]. A study by Daud et al. analyzed the changes in TLR expression in women that either cleared or not HPV16 infection and showed that virus clearance was associated with an increase in TLRs including TLR9, while the persistence correlated with a decrease in TLR3, TLR7/8, and TLR9 expression [257]. In all, we assume that viruses have developed an escape mechanism based on the interaction of virus particles with TLR9 (reviewed in [188]). This phenomenon is not only restricted to chronic infections associated to cancer but also to non-viral-induced cancers. TLR9 has also been demonstrated to be expressed in solid tumors including hepatocellular carcinoma, breast, prostate, and lung cancers as well as hematologic malignancies, especially B cell lymphoma and multiple myeloma [55, 258, 259]. Although TLR9 expression has been shown to be associated to several solid tumors, the exact cell type in a given immunohistochemical block needs to be microdissected to fully understand the implication of the receptor in a given cell type.

TLRs and especially TLR9 are differentially regulated depending on the type of cancer. Their dysregulation might impact on the ability of TLR ligands to drive cell proliferation or apoptosis. TLR agonists have been used in a plethora of xenograft models or in vitro studies to determine their pro- or antitumor role on tumor growth. On one hand, CpG-mediated targeting of TLR9 in xenograft models of human ovarian carcinoma, small cell lung cancer, colon cancer, lung adenocarcinoma, and neuroblastoma, led to a decrease of cell proliferation and in the end tumor size when compared to controls [56, 260, 261]. TLR9 agonists were shown as well in vitro to decrease human colon cancer cell proliferation and survival.

In contrast, TLR9 engagement has been shown in other studies to induce proliferation and invasiveness of cancer cells. Triggering of TLR9 using CpG DNA promoted tumor invasion [55] and migration [58] in breast and prostate cancers [262] in in vitro tumor models. This was associated to increased NF κB [58] and matrix metalloproteinase activities, especially MMP13 [55]. Finally, stimulation of TLRs has been shown to be either proapoptotic [263, 264] or anti-apoptotic [265] depending on the TLR, the cell type, and the metabolic condition of the cell. It was for instance shown that treatment with TLR9 ligand increases the survival of nude mice with experimentally induced brain tumors by inducing apoptosis [263]. However, the exact mechanisms remain unclear.

Triggering of extrinsic TLR9 activity in tumor microenvironment

Besides their intrinsic effect on the tumor cells, TLR9 ligands display also extrinsic activities on the microenvironment that could favor or fight against tumor development. The role of TLRs in tumor angiogenesis is quite diverse just as cancer is itself. Cyclooxygenase-2 (COX-2) is known to play a crucial role in *Helicobacter pylori*-associated gastric cancer. *H. pylori* was shown to act through TLR2 and 9 to activate the MAPK cascade, leading to COX-2-dependent prostaglandin E2 (PGE2) release and thereby contributing to cancer cell invasion and angiogenesis [266]. On the other hand, TLR9 ligands can also cause antitumor activity, interfering with angiogenesis. For instance, IMO TLR9 agonist inhibited vessel formation and tumor growth [267] by a mechanism still not well understood.

Based of their ability to stimulate human pDC and B cell, TLR9 agonists could provide strong humoral and Th1 cellular responses. Interestingly, the efficacy of CpG in mouse melanoma B16F10 model was shown to be dependent on the cross-talk between pDCs and specific subsets of cDCs [268]. However, lack of TLR9 responsiveness of pDC in breast, ovarian [269], SCCHN [270], and NSCLC [271] cancers and several virus infections is a well-documented

observation (discussed in [188]). This shall be taken into consideration in the prospect of using TLR9 ligands in immunotherapy. pDCs from HCV/HIV-infected patients or isolated from ovarian or breast tumors display weak production of IFNα after ex vivo exposure to TLR9 agonists [269, 272–274]. The mechanism involved in lack of TLR9 responsiveness in cancers was sometimes associated to a decreased expression of TLR9 [270] but is still not well understood. Similarly, in vitro infection of B cell by EBV (lymphoblastoid cell line) led to TLR9 downregulation and decreased cytokine response (TNFα, IL8) to CpG DNA [223]. Due to in vitro limitation, the effect of TLR9 downregulation on plasma cell differentiation and humoral responses was not assessed.

RLR

RIG-I and MDA5 are two RNA helicases now considered as a first line of defense against viral dsRNA [72]. MDA5 was first described as a melanoma differentiation-associated gene [61]. It is expressed at low abundance in many normal tissues, whereas expression is induced in normal and cancer cells by type I IFN [275]. MDA5 was shown to display proapoptotic activities when expressed ectopically at high levels [275, 276]. Mutational analysis demonstrated that both CARD and helicases domains were required for full growth inhibition [61]. However, it was unclear how MDA5 was driving apoptosis on its own. Its triggering by complexed Poly[I:C] was indeed recently shown to induce the formation of autophagy vesicles (autophagosomes) and NOXA-dependent cell death [277]. Furthermore, upon apoptotic stimuli, MDA5 is cleaved by caspases, thereby separating the CARD domains from the helicase domain. While MDA5 localizes in the cytoplasm, the helicase-containing fragment is found in the nucleus where it accelerates FasL-induced DNA degradation [276].

While RLR ligands are not yet under clinical trial, in vitro data strongly suggest that triggering MDA5 or RIG-I would represent a promising approach in cancer treatment. Because recognition of 5′-triphosphate RNA (5′triP-RNA) by RIG-I is mostly independent of the RNA sequence and gene silencing is not inhibited by the presence of a 5′triP moiety, both biological activities can be induced by one short dsRNA molecule. Poeck et al. demonstrated that systemic administration of 5′triP-siRNA designed to specifically silence *bcl2* and activate RIG-I, triggers melanoma tumor growth inhibition reflected by massive melanoma tumor apoptosis [278]. Downregulation of bcl2 and immune activation via RIG-I (type I IFN, CXCL10, and MHC class I production) lead to apoptosis of melanoma cells [278]. 5′ triP-RNA triggering preferentially induced apoptosis in tumor cell, whereas primary cells are less susceptible due to an intact Bcl-xL counter-regulatory pathway [279]. This response requires recruitment of NK cell at tumor site and is associated with the induction of systemic Th1 cytokines (IFN α, IL-12p40, and IFN γ) [278]. Similarly, ovarian cancer cells treated with complexed Poly[I:C] reacted with enhanced expression of HLA-class I, release of cytokines (CXCL10, IL-6, and type I interferon), as well as tumor cell apoptosis [280]. DC engulfed MDA5-activated cancer cells, became activated, and subsequently provided a proinflammatory milieu promoting cytolytic activity and IFN γ secretion by NK cells [280].

RIG-I has been involved as well in antitumor activity in acute myeloid leukemia (AML). RIG-I was shown to be highly upregulated in all-trans retinoic acid (ATRA)-induced terminal granulocytic differentiation of APC cell line [281]. RIG-I-deficient mice displayed an abnormal Gr-1^{hi}/Mac-1^{hi} granulocytic compartment in peripheral blood leukocytes [74], which would evolve to a CML-like phenotype. Furthermore, in vitro colony assays revealed an intrinsic defect of RIG-I-deficient myeloid progenitors to proliferate and differentiate suggesting that RIG-I exerts a critical role in basic regulatory mechanism controlling cell growth and survival [74]. The same group recently published that ATRA-induced RIG-I induction exerts a critical feedback effect, similar to what is seen using type I IFN or ATRA, to restrain cellular proliferation of AML in vivo and in vitro [282]. RIG-I effect is mediated by a strong activation of STAT1 and interferon-stimulated gene induction (such as ICSBP) [282].

Altogether, those data provide strong proof of concept that the engagement of MDA5 (ovarian cancer) or RIG-I (melanoma, AML) exhibits anticancer effect through two essential and interconnected effects: (a) inhibition of proliferation and induction of apoptosis of tumor cells and (b) mobilization of antitumor immune responses. Those RLR further represents promising ligands to use in cancer therapy.

Concluding remarks

Agonists of TLR and other innate sensors are actively pursued for therapeutic applications in oncology based on three major rationales. First, TLR agonists are potent immunomodulators that can strongly stimulate innate immunity and initiate long-lasting adaptive immunity through systemic activity. The more advanced molecules developed in clinic along with this rational mainly included (a) whole bacteria and defined TLR2/4 ligands and (b) TLR9 ligands. Second, certain ligands have demonstrated antitumor activity based on direct tumoricidal activity on tumor cells, these mainly included TLR3 and TLR7 ligands. Third, vaccine companies are developing TLR ligands as vaccine adjuvant based

on local delivery together with formulated tumor antigen leading to local activation and DC maturation resulting in initiation of long-lasting adaptive immunity. Many TLR ligands are developed along this line; the most advanced in clinic include TLR9 and TLR4 ligands. Finally, recent advances in the field of RLR lead to very exciting preclinical data promoting RLR ligands to the level of TLR agonists as potential immunotherapeutic agents in cancer treatment.

For all these lines of investigations, the development has been held back by the limited transposition of mouse preclinical models to human clinic. For example, the wide distribution of TLR9 in mouse APC compared to its limited expression to pDC and B cells in human makes the mouse preclinical models for both vaccine development and systemic delivery not predictable.

For clinical development, we also have to take into consideration detrimental activities of TLR ligands such as pro-tumoral activity through indirect inflammatory process or protective activity against chemotherapy through direct pro-survival effect. The clinical development of these ligands aimed at treating patients with advanced tumors and potential immune defect. Importantly, it will be critical for the future clinical trials to (a) define the expected mechanisms of action and (b) determine the functional competence of the identified TLR target cells in patients (DC in periphery or at tumor sites). Based on these understandings, it is expected that TLR ligands will undoubtedly be successfully developed in clinical trials and will allow to reach therapeutic responses in cancer patients through elicitation of long-lasting antitumor immunity.

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