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Critical Reviews in Oncology/Hematology 94 (2015) 31–44

CRITICAL REVIEWS IN
Oncology
Hematology
Incorporating Geriatric Oncology

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MGN1703, an immunomodulator and toll-like receptor 9 (TLR-9) agonist: From bench to bedside

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Accepted 9 December 2014

Contents

Abstract

The adaptive immune system has been the main focus of immunological strategies in oncology with only more recent approaches targeting innate immunity. Endosomal toll-like receptors (TLR-7, TLR-9) activate innate immune responses by signaling damage-associated molecular patterns (DAMP) from decaying tumor cells. This has led to the development of DNA-based TLR-9 agonists, which induce antitumor activity through innate and subsequent adaptive immune responses. Early clinical trials with CpG-ODN as TLR-9 agonists were associated with unfavorable tolerability and narrow clinical efficacy, leading to failure in pivotal trials. dSLIM®, the active ingredient of MGN1703, is a DNA-based, radically different molecular alternative to CpG-ODN, which results in genuine antitumor immunomodulation. Preclinical and

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http://dx.doi.org/10.1016/j.critrevonc.2014.12.002

clinical studies of MGN1703 have confirmed that this TLR-9 agonist has therapeutic potential in a variety of solid tumors, while long-term treatment with high doses was very well tolerated. A pivotal trial of first-line maintenance treatment with MGN1703 in patients with metastatic colorectal cancer is underway.

Keywords: Innate immune response; Toll-like receptor 9; dSLIM®; MGN1703; Anti-cancer immunomodulation; Colorectal cancer

1. Introduction

There is a growing recognition that the immune system plays a key role in the regulation of tumor growth and development. Components from both the innate and adaptive immune systems are involved and cell types such as natural killer cells (NK), natural killer T cells (NKT), dendritic cells (DC), macrophages, polymorphonuclear leukocytes, mast cells, myeloid-derived suppressor cells, T lymphocytes and B lymphocytes all have roles to play [1,2]. Some of these components inhibit the growth of cancer cells, resulting in their eventual death in a process known as immunosurveillance [3,4], however, others promote tumor development leading to a complicated balancing act between pro- and antitumor immunity (Table 1) [1,3].

This dual, and seemingly contradictory, functionality of the immune system was initially described by the immunoediting hypothesis, which included three phases: elimination, equilibrium and escape [5]. During the elimination phase, both an innate and adaptive immune response is triggered which may eliminate the tumor, whilst during the equilibrium phase, those cancer cells which were not eliminated persist in dynamic interaction with the immune system. Finally, the escape phase represents a period when tumor cells evade elimination by the immune system and the cancer progresses [5]. More recently the cancer stem cell, or the tumor initiating cell (TIC) hypothesis model has been described. Here, a small subfraction of TIC form a homogeneous stem cell-like population driving tumor maintenance and metastatic formation [6]. Evidence also suggests that TIC are not static, but can evolve and acquire additional genetic mutations and epigenetic modifications resulting in subclones, which consist of functionally different cells. Such functional diversity could also contribute to drug resistance, with chemotherapy leading to a selection of cells conferring survival advantages [6].

Table 1
Innate and adaptive immune cells involved in regulating tumor growth [1,3].

Stimulate cancer growth	Inhibit cancer growth
Innate immune cells	
Neutrophils	DC
Macrophage (M2)	Macrophage (M1)
Myeloid-derived suppressor cells	NK cells
Adaptive immune cells	
Th2 CD4+ T cell	Cytotoxic CD8+ T cell
CD4+ T regulatory cell	Th1 CD4+ T cell
	Th17 CD4+ T cell
Bridge both innate and adaptive systems	
NKT (type 2)	NKT (type 1)

DC, dendritic cell; NK, natural killer; NKT, NK T cells; Th, T-helper.

A number of immunologic approaches to treat cancer are based around activation of the endogenous adaptive immune response. One example is passive immunization, or adoptive cell therapy (ACT), which involves the transfusion of autologous or allogeneic T cells into a patient [7]. A variety of ACT methods are under investigation and the most developed of these is termed tumor-infiltrating lymphocyte (TIL) therapy [8]. Tumor-specific T cells are harvested from a biopsy specimen and cultured in vitro in the presence of interleukin (IL)-2 to promote growth. Following chemotherapy and/or radiotherapy to eradicate circulating lymphocytes, the patient receives a transfusion of the cultured T cells which can then induce a concerted antitumor response [7]. TIL therapy has shown promise in the treatment of metastatic melanoma [9]. Other methods of ACT are currently in development and clinical trials are ongoing in a wide range of cancers [10].

Active immunization through vaccination is another approach and encouraging results have been seen in prostate cancer with the vaccine sipuleucel-T. This immunotherapy product is prepared using a patient's own antigen presenting cells (APC), which are harvested and cultured ex vivo with a prostate antigen-containing fusion protein PA2024. T-cell immunity to the prostate antigen is induced via inclusion of granulocyte-macrophage colony-stimulating factor [11,12]. Two studies demonstrated that immunization with sipuleucel-T extended median overall survival (OS) in comparison to placebo [11,12], which led to Food and Drug Administration (FDA) approval of this cancer vaccine.

A third approach involves blocking regulatory checkpoints such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) or the programmed death-1 pathway and associated programmed death ligand 1 (PD-L1), which all limit the T-cell response and are therefore targets for cancer immunotherapy [13,14]. Fully human monoclonal antibodies directed against CTLA-4 have been developed, with the most widely studied being ipilimumab [15]. In patients with metastatic melanoma, this treatment has been reported to extend median OS [16], and ipilimumab has consequently been approved by the FDA for this indication. It is also being investigated in a variety of other cancers [15]. Development of a number of similar monoclonal antibodies is currently

ongoing, with nivolumab and lambrolizumab being the most advanced [13].

In addition to these three approaches, research is ongoing to investigate aspects of the innate immune response as potential targets for cancer therapies. During tumor cell death, a variety of molecules are dispersed that act as signals to the innate immune system warning of damage or danger [2]. Known as damage-associated molecular pattern molecules (DAMP), they are recognized by a variety of receptors, including the toll-like receptors (TLR) found on innate immune cells, resulting in activation of a cancer-associated inflammatory response [2,17]. While the interplay between cancer-derived DAMP and TLR is complex, this represents an alternative developmental area for cancer immunotherapy options.

More recently, several innate lymphoid cells have been identified including NK cells, which can distinguish stressed cells from healthy cells via recognition of up-regulated activating ligands. Such recognition results in the secretion of pro-inflammatory cytokines or can promote direct NK-cell mediated cytotoxicity [18]. Invariant NKT (iNKT) cells have been shown to function as both innate cells and memory cells via expression of a semi-variant T cell receptor. iNKT cells can be indirectly activated by high amounts of pro-inflammatory cytokines and in this setting resemble NK cells and produce a T-helper (Th)1-type cytokine response [18].

This review focuses on the role TLR play as sensing modulators of the innate immune system and the therapeutic potential of targeting one specific member of this protein family, TLR-9, with the TLR-9 agonist MGN1703 which has now entered Phase III trials in the first-line/maintenance setting in patients with metastatic CRC (Immunomodulatory MGN1703 [IMPALA]; ClinicalTrials.gov Identifier: NCT02077868).

2. Damage-associated molecular pattern molecules (DAMP)

One of the key processes of the non-infectious inflammatory response involves the release of DAMP, which may be produced in response to a variety of stimuli including cellular injury, necrotic cell death, ischemia and other stress factors [19]. A cascade of signaling events, termed signal 0 to signal 5, is triggered by the presence of DAMP [17]. The signal 0 events induce the early innate immune responses through a variety of receptors and ligands, which facilitate the signaling through triggers of inflammation, such as cytokines and chemokines, ultimately culminating in a full immune response [17,19].

As discussed, a host of different inflammatory cells are known to be activated in the presence of malignant cells [17,20]. However, if this acute inflammatory response is insufficient to fully destroy the developing tumor, a dysregulation of the immune system can occur, resulting in a chronic inflammatory response typified by production of large numbers of certain innate immune cells that can ultimately promote the growth and progression of cancer [17,20]. It has been suggested that DAMP-like re-arranged membrane components, nuclear-, cytoplasmic-, and mitochondrial proteins as well as DNA from damaged mitochondria and cleaved chromatin, are released following tumor cell decay, and contribute to this dysregulation. In turn, this may help to perpetuate a favorable environment for tumor proliferation [17]. Specific DAMP appear to be associated with certain types of malignancy. Typical examples include the high mobility group box 1 and S100 proteins which have been linked to cancers of the breast, liver, oral cavity, prostate and genitalia [21].

Generally found on the surface of micro-organisms, pathogen-associated molecular pattern molecules (PAMP) allow the innate immune system to recognize and respond to an invading pathogen [17]. Similar to DAMP, and in addition to being part of the infectious immune response, some PAMP are also associated with a range of different types of cancer [21].

Both DAMP and PAMP may be bound by the same transmembrane pattern recognition receptors (PRR), in particular by various members of the TLR family, albeit by different mechanisms [21,22]. Binding of these molecules to TLR results in the induction of TLR signaling pathways, thereby promoting an inflammatory response [22]. However, in the presence of tumor cells, dysregulation of TLR signaling can also create an environment conducive for tumor growth [23]. Consequently, TLR and TLR signaling is an increasing area of interest for the development of new antitumor agents.

3. Toll-like receptor 9 (TLR-9)

TLR are a key component of the innate immune system and are essential for the recognition of PAMP and/or DAMP. TLR belong to the type 1 transmembrane glycoproteins. Currently ten human TLR (TLR-1 to TLR-10) have been identified, which differ according to their cellular location and varied activating ligands [24,25]. This section will focus on TLR-9, which is the TLR with the narrowest tissue distribution. In resting human immune cells, it is only expressed on B cells and plasmacytoid dendritic cells [26]. Upon cell activation, TLR-9 can also be found in neutrophils, monocytes and T cells [27].

TLR-9 is activated by non-methylated CG motifs from nucleotide sequences. Non-methylated CG motifs are common in bacterial and viral genomes, but occur infrequently in mammalian DNA [28,29]. Where CG motifs do occur in mammalian DNA, they tend to be methylated [28]. The exception is mitochondrial DNA (mtDNA), since mitochondria are evolutionary endosymbionts that were derived from bacteria [30]. Injury/trauma releases mitochondrial DAMP – i.e., non-methylated CG motifs – into the circulation. Given that they are derived from mtDNA, these DAMP have evolutionarily conserved similarities to bacterial PAMP [31].

Consequently, the non-methylated CG motifs cause a strong immune response, which is mediated primarily by TLR-9 [28,30–33]. To date, TLR-9 appears to be the only TLR that is activated by non-methylated CG motifs from mitochondrial DAMP.

The activation of TLR-9 by non-methylated CG motifs was first demonstrated by studies performed in a murine model [28]. When challenged with CpG-oligodeoxynucleotides (CpG-ODN), TLR-9-deficient mice did not induce typical immune responses seen in wild-type mice [28]. Increased proliferation of splenocytes or production of inflammatory cytokines from macrophages were not observed in the TLR-9-deficient mice, even after further stimulation with interferon (IFN)-gamma. Additionally, the maturation of DC did not occur, there was a lack of expression of surface molecules CD40, CD80, CD86 and major histocompatibility complex (MHC) class II, and the T-helper type-1 response was absent. These findings demonstrated that the presence of CpG-ODN in a cell triggers a mechanism to initiate binding with TLR-9 and ultimately signal transduction.

TLR-9 is located in the endoplasmic reticulum, primarily in plasmacytoid DC (pDC), where the functional role of this receptor has been proven. TLR-9 is also expressed on the majority of innate and adaptive (CD4+, CD8+, NKT and γδ T) effector cells [27], where its function is less clear, and in B cells [24,34,35]. Transport of CpG-ODN internally into the endosomal/lysosomal compartment of the cell occurs via endocytosis in human resting cells [36,37]. It is thought that an endoplasmic reticulum membrane protein, UNC93B1, then recruits TLR-9 and delivers it to the same lysosomal compartment, where it binds directly to CpG-ODN [36–39]. Following this binding, TLR-9 engages with the myeloid differentiation primary response 88 adaptor protein, which triggers a distinct signaling pathway [24,36]. Transduction molecules activated in this cascade include interleukin-1R-associated kinases and tumor necrosis factor receptor-associated factor 6 [40]. In turn, this stimulates both the nuclear factor kappa B and IFN regulatory factor-7 pathways, resulting in production of pro-inflammatory cytokines and type I IFN, respectively [29,40]. An overview of the signaling pathway and immunologic response following TLR-9 activation is summarized in Fig. 1.

TLR are also involved in linking the innate to the adaptive immune response; although they are classified as transmembrane PRR of the innate immune system, they can activate classes of cells associated with the adaptive immune system [41]. The development of mature APC from immature DC is facilitated by TLR. This results in induction of costimulatory molecules such as CD40, CD80 and CD86, which

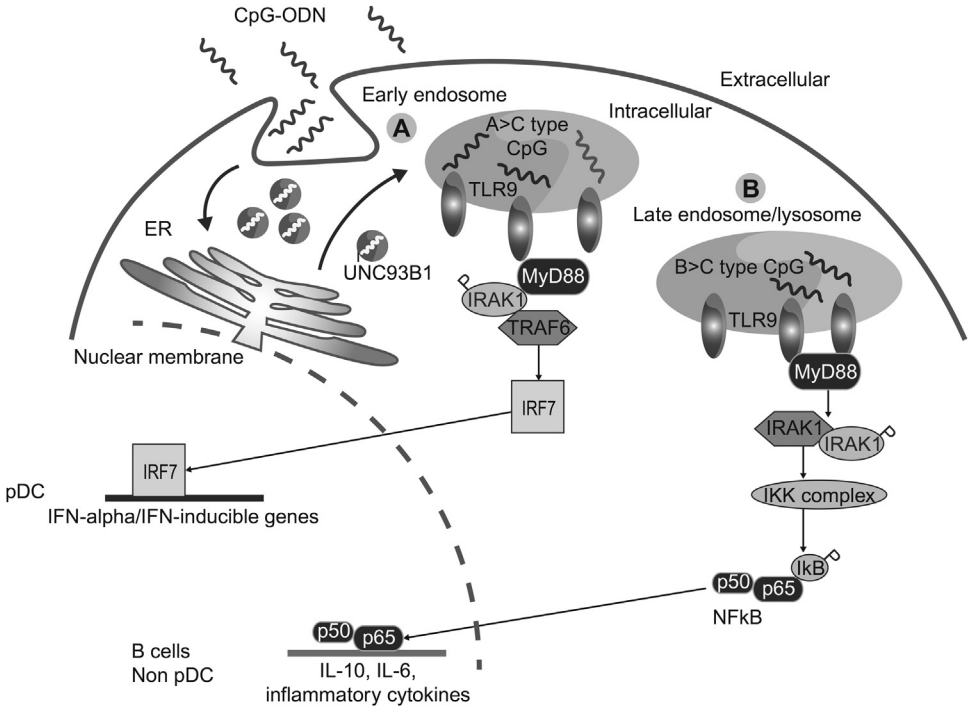

Fig. 1. Signaling pathway following TLR-9 activation [26]. On internalization, CpG-ODN encounter TLR-9 located in the endolysosomal compartment. In pDC, spatiotemporal regulation impacts downstream signaling cascades and the resulting cytokine profile. (A) Long-term encounter of Type A CpG-ODN with TLR-9 occurs in early endosomes. Recruitment of the adapter protein MyD88 by activated TLR-9 initiates downstream signaling via IRAK/TRAF and activation of IRF7, resulting in high IFN-alpha levels. (B) In contrast, Type B CpG-ODN is quickly transferred to lysosomal vesicles. Here, encounter with TLR-9 culminates in activation of the NF-κB pathway and secretion of a number of inflammatory cytokines. CpG-ODN, Oligodeoxynucleotide(s) containing one or more non-methylated CG motifs; IRAK, interleukin-1R-associated kinase; IRF7, IFN regulatory factor 7; MyD88, myeloid differentiation primary response 88 adaptor protein; NF-κB, nuclear factor kappa B; pDC, plasmacytoid dendritic cell; TLR-9, toll-like receptor 9; TRAF, tumor necrosis factor receptor-associated factor.

are components of the T-cell activation process [24]. In addition, T-cell differentiation into CD4+ T-helper type 1 or CD8+ cytotoxic lymphocytes is directed by TLR-induced cytokines [24]. The involvement of TLR in both the innate and adaptive immune response suggests that they may be useful targets for immunotherapy.

4. Toll-like receptor 9 (TLR-9) agonists in tumor immunotherapy

Bacteria or bacterial extracts associated with non-methylated CG motifs have long been known to possess antitumor activity, as well as to have a powerful effect on the immune system [42–44]. In addition, it is known that during tumor cell death, mtDNA and mitochondrial formyl peptides are released, which may act as DAMP and potentially result in the dysregulation of TLR-9 [2]. Therefore, while complex, there does appear to be a role of TLR-9 in the development of some tumors. For example, a study by Eiró et al. found a potential protective role of TLR-9 expression against malignant transformation in the colorectal mucosa: TLR-9 expression was decreased in hyperplastic and villous polyps from patients who developed colorectal cancer (CRC) [45]. TLR-9 has also been shown to be associated with certain other tumors [26].

As a consequence, TLR-9 has become a target of investigation for various malignancies [46,47]. In this regard, development of synthetic ODN containing non-methylated CG motifs has been the cornerstone of research into TLR-9 agonists for tumor immunotherapy [46,48]. As native DNA is subject to degradation by nucleases, synthetic CpG-ODN may be modified to enhance stability. For example, one approach is for the normal phosphodiester component of the backbone of DNA to be replaced by a nuclease-resistant phosphorothioate (PT), which increases the half-life from a few minutes to around 2 days [48]. CpG-ODN can be classified into three separate classes with different structural characteristics and differentially enhancing antigen-specific humoral and cellular immune responses [49,50]: Class A are strong inducers of IFN-alpha from pDC but very poor B cell activators, Class B are potent stimulators of B cell proliferation with poor induction of pDC IFN-alpha secretion, while Class C CpG-ODN exhibit moderate properties from both Class A and Class B [49].

To date, the most widely studied CpG-ODN TLR-9 agonist has been PF-3512676, which is a Class B, single-stranded agent (also known as ProMune, CpG-7909 or ODN2006). Investigation of PF-3512676 monotherapy in a variety of cancers has shown mixed results [47,51–55]. In 41 patients with chronic lymphocytic leukemia no clinical response was reported following PF-3512676 given as an intravenous (i.v.) infusion (1.05 mg/kg) or subcutaneously (0.45 mg/kg) [55]. Similarly, 23 non-Hodgkin lymphoma patients administered with i.v. PF-3512676 at doses up to 0.64 mg/kg showed no clinical response at day 42, although two patients demonstrated late response [52]. Some activity was seen in a phase II trial of 20 patients with metastatic melanoma, who received 6 mg PF-3512676 subcutaneously [53]. Two partial responses (PR) were reported, one of which continued for >140 weeks, along with an additional three patients achieving stable disease (SD). Similarly, some activity was seen in a phase I dose-escalating trial in patients with cutaneous T cell lymphoma (51). Twenty-eight patients received up to 0.36 mg/kg of subcutaneous PF-3512676 and three complete responses (CR) were reported along with six PR. Finally, 39 patients with stage IV renal cell carcinoma receiving subcutaneous PF-3512676 at doses up to 0.81 mg/kg in a phase I/II study reported two PR [54].

Combination trials of PF-3512676 administered in conjunction with standard chemotherapy regimens as first-line treatment in advanced non-small-cell lung cancer have not shown improvement over the monotherapy trials mentioned above. A phase III study comparing PF-3512676 with or without paclitaxel/carboplatin reported no improvements in median OS (10.0 months versus 9.8 months; $P = 0.56$) or median progression-free survival (PFS) (4.8 months versus 4.7 months; $P = 0.79$; [56]). A second, similar phase III study comparing PF-3512676 with or without gemcitabine/cisplatin also showed little difference in median OS (11.0 months versus 10.7 months; $P = 0.98$) and no difference in median PFS (both groups were 5.1 months) [57]. In addition, both studies revealed a higher proportion of adverse events (AE) $\geq$ grade 3 in patients receiving PF-3512676. Due to the lack of significantly improved efficacy and increased toxicity seen with PF-3512676, both trials were terminated early due to an unfavorable risk-benefit profile [56,57].

In order to improve efficacy and reduce toxicity, structural changes were made to CpG-ODN, including linking two CpG-ODN with their 3′-ends and through the inclusion of novel synthetic immunostimulatory motifs [26]. This resulted in the development of the second-generation TLR-9 agonist, IMO-2055. A phase I study administering IM0-2055 plus gemcitabine/carboplatin to 18 patients with solid tumors reported a single case of PR [58]. A second phase Ib trial combining IMO-2055 with 5-fluorouracil/cisplatin/cetuximab in 13 patients with recurrent or metastatic squamous cell carcinoma of the head and neck reported three patients achieving PR and nine patients with SD [59]. However, as serious AE occurred in eight patients, four of which were deemed to be related to IMO-2055, and one patient died, the trial was stopped prematurely. Additionally, a phase II trial in this indication, did not meet its primary endpoint of improved PFS following treatment with IMO-2055 in combination with cetuximab compared to treatment with cetuximab alone [60].

Given the results described above, it is unsurprising that at the present time, no CpG-ODN has been approved in an oncology indication. This demonstrates the need for the development of an alternative, next generation TLR-9 agonist for cancer therapy. In this respect, the family of double-stem loop immunomodulators (dSLIM), such as MGN1703, has shown promise. These agents also include CG motifs within

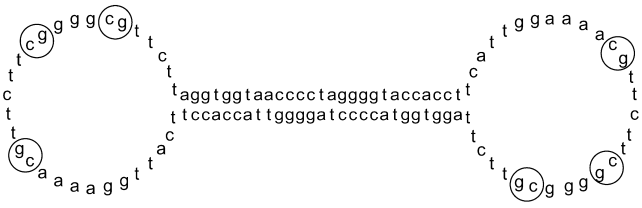

Fig. 2. Descriptive secondary structure of MGN1703 [62]. MGN1703 is a member of the dSLIM family exhibiting a double stem of 28 base pairs and two loops with 30 nucleotides – each containing three CG motifs (circled). dSLIM, double-stem loop immunomodulator.

their structure. However, they are structurally distinct from CpG-ODN; they are double-stranded rather than linear and include a natural phosphodiester, rather than a phosphorothioate backbone [47,61,62]. It has been proposed that this novel structure may improve activity and lead to improved toxicity versus earlier TLR-9 agonists. The following section describes the development of MGN1703, including a summary of preclinical and clinical results reported with the agent.

5. Preclinical characteristics of MGN1703

5.1. Structure and physical characteristics of MGN1703

MGN1703 is a small DNA molecule containing 116 nucleotides [61]. The molecule has a covalently closed dumbbell-shaped structure due to a stretch of reverse complementary DNA forming a 28 base pair double-stranded midsection, flanked at either end by two single-stranded loops, each containing 30 nucleotides (Fig. 2). Natural phosphodiester bonds maintain the non-branched structure and there are no unnatural components present in the molecule. Sequences of each loop comprise three effective non-methylated CG motifs [61,62].

5.2. Biological effects of MGN1703 on cytokines and immune cells

The structure and sequence of MGN1703 results in a unique immunomodulation profile with regard to activation of cells and production of proteins of the immune system [61–63] as specified below. An overview of the mechanism of action of MGN1703 is shown in Fig. 3.

The effect of MGN1703 on cellular activation of a variety of components within the peripheral blood mononuclear cell (PBMC) pool was demonstrated by a significantly increased expression of specific surface activation markers [61]; CD86 was up-regulated on monocytes, myeloid DC (mDC) and B cells, CD40 expression was increased on pDC, higher levels of HLA-DR (MHC class II) were detected on pDC and mDC, CD169 became up-regulated on monocytes, and CD69 levels increased on NK, NKT cells and T cells (Table 2). In PBMC, MGN1703-dependent CD69 up-regulation on NK cells occurred at the same time as NK-cell mediated cytotoxicity increased [61]. This is important for the anti-cancer effect of MGN1703, since NK cells are responsible for selectively eliminating harmed cells by inducing apoptosis.

MGN1703 has also been shown to significantly increased the release of a variety of cytokines and chemokines from PBMC. Secretion from PBMC occurred during the first 48 h of exposure and included IFN-gamma-induced protein 10 (IP-10), IFN-alpha, macrophage inflammatory protein (MIP)-1-alpha, MIP-1-beta, IL-6, IFN-gamma, monocyte chemotactic protein (MCP)-1 and IL-8 (Table 2) [61]. Expression of IL-12p70, monokine induced by gamma-interferon (MIG) and tumor necrosis factor (TNF)-alpha did not appear to be significantly affected by MGN1703 (Table 2).

In terms of establishing the specific cellular targets of MGN1703, isolated TLR-9-positive pDC showed increased secretion of IFN-alpha, MIP-1-alpha, MIP-1-beta, IL-6, TNF-alpha and IL-8 following MGN1703 stimulation. Additionally, activation by MGN1703 of isolated TLR-9-positive B cells led to increased expression of MIP-1-alpha, IL-6, TNF-alpha and IL-8 compared with incubation with medium [61]. The direct activation of isolated TLR-9-positive cells, as well as absence of a cellular activation profile evoked by MGN1703 in PBMC populations depleted of either TLR-9-positive cell type, strongly point to MGN1703 as a TLR-9 ligand. This pharmacological action has been corroborated in a study of an MGN1703 derivative in which all CG motifs in both loops were switched to thymidine-guanine (TG) nucleotide sequences. This non-CG MGN1703 no longer activated pDC nor a reconstructed signaling cascade in a model cell line with a TLR-9-triggered eGFP expression (ELAM41 cells) [61]. Furthermore, the activation of ELAM41 cells by MGN1703 was inhibited by chloroquine, a lysosomotropic agent that prevents endosomal acidification – a process necessary for TLR-9 signaling [65].

Activation of both the innate and adaptive immune responses is known to be associated with increased expression of type I IFN, including IFN-alpha, from pDC [66,67]. In addition, reports show a link between CD169 expression on monocytes and the induction of a type I IFN response [68,69]. The data described here show that exposure to MGN1703 significantly increased secretion of IFN-alpha in pDC and expression of CD169 in monocytes, suggesting that MGN1703 has a role to play in activation of components of both the innate and adaptive immune systems via the IFN-alpha pathway (Fig. 3). This is important, since activation of both innate and adaptive immune response is believed to be required for an effective antitumor response [70].

5.3. Influence of molecule structure on the immunomodulatory effect of MGN1703

Stem-loop constructs varying in number and distribution of CG motifs, and stem and loop size, have been studied for their immune-stimulatory potential [63]. Notably, molecules

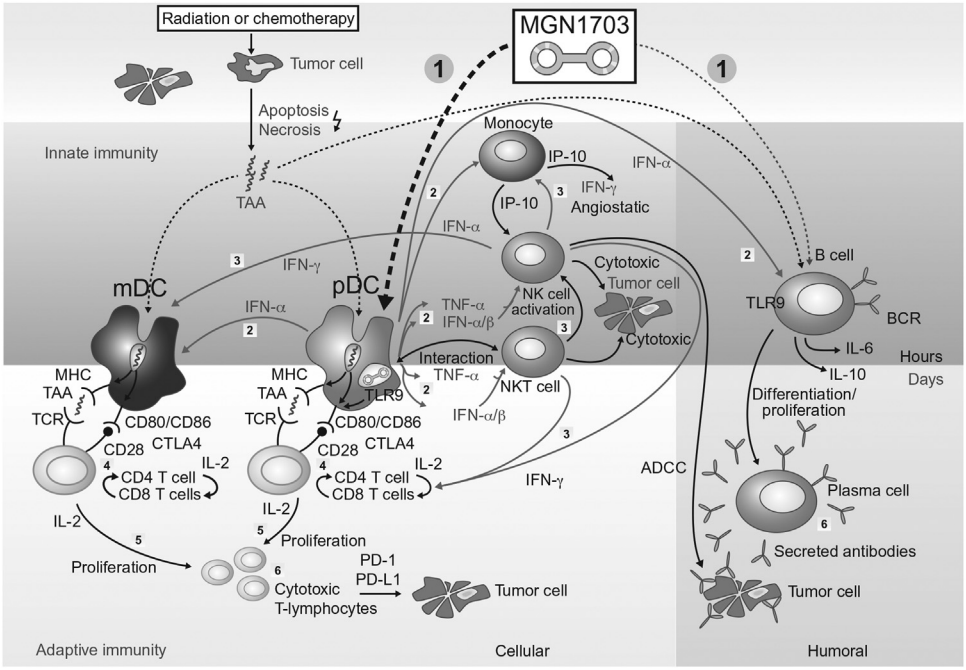

Fig. 3. Mechanism of action of MGN1703 [64]. (1) MGN1703 activates TLR9-bearing pDC and B cells. (2) IFN-alpha, secreted by pDC, initiates broad activation of the innate and adaptive immune system: NK cells and NKT cells are activated for cytotoxicity against tumor cells. Monocytes and B cells are activated. (3) Monocytes secrete cytokines and chemokines: IP-10 is chemotactic for NK cells and inhibits tumor growth through its angiostatic properties. IFN-alpha, together with IFN-gamma from NK and NKT cells, activates mDC. Furthermore, IFN-gamma regulates the differentiation of T cells. (4) pDC and mDC take up TAA released from tumor cells decaying due to prior therapies; TAA are presented to T cells. (5) CD4+ helper T cells and CD8+ cytotoxic T cells for tumor cell elimination are generated by proliferation. (6) IFN-alpha amplifies activation of B cells, resulting in antibody secretion against antigens on tumor cells which can be eliminated by NK cells via ADCC. See also Table 1. Republished with permission of Springer-Verlag Berlin/Heidelberg, Schmoll HJ et al., Maintenance treatment with the immunomodulator MGN1703, a Toll-like receptor 9 (TLR9) agonist, in patients with metastatic colorectal carcinoma and disease control after chemotherapy: a randomised, double-blind, placebo-controlled trial. J Cancer Res Clin Oncol 2014;140(9):1615–24; permission conveyed through Copyright Clearance Center, Inc. ADCC, antibody-dependent cell-mediated cytotoxicity; BCR, B cell receptor; CTLA4, cytotoxic T lymphocyte-associated protein 4; IFN, interferon; IL, interleukin; IP-10, IFN-gamma-induced protein 10; mDC, myeloid dendritic cells; MHC, major histocompatibility complex; NK cell, natural killer cell; NKT cell, natural killer T cell; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; pDC, plasmacytoid dendritic cells; TAA, tumor associated antigens; TCR, T cell receptor; TNF, tumor necrosis factor.

with only one loop, CG motifs in only one loop or those with CG motifs in the double-stem, diminished the increase in IFN-alpha secretion from human PBMC or IL-12p40 from murine spleen cells compared with MGN1703 (Fig. 4A–C). Furthermore, the length of the stem and size of the loop was critical; the MGN1703 composition with the 28 base pair double-stem and loop containing 30 nucleotides was identified as the most effective. This was shown by an increase of IFN-alpha expression from human PBMC (Fig. 4D). In keeping with these findings, other reports have also described a dependence on the secondary structure of the molecule [71,72].

5.4. Differential effects of MGN1703 versus CpG-ODN

MGN1703 differs from CpG-ODN not only in terms of sequence and structure, but also with respect to pharmacological parameters. The TLR-9-positive ELAM41 cell system was used to measure potency of MGN1703 versus members of the CpG-ODN classes: A, B and C [61]. MGN1703 developed an EC_{50} of 0.1 μM compared with EC_{50} of 1.5 μM (Class A ODN2216), 0.4 μM (Class B ODN2006) and 0.8 μM (Class C M362) indicating that MGN1703 is more potent than CpG-ODNs in this system (Fig. 5A).

Furthermore, immunological responses in PBMC stimulation of cytokine secretion and activation of immune cells differed between MGN1703 and CpG-ODN [61]. Exposure to MGN1703 and ODN2216 resulted in secretion of IFN-alpha and IP-10 while MGN1703, ODN2216 and ODN2006 stimulated IFN-gamma production (Fig. 5D and E). Activation of monocytes and NK cells was high after exposure to either MGN1703 or ODN2216, whereas ODN2006 demonstrated very limited activation of either cell type (Fig. 5B and C) [61]. In contrast, activation of PBMC with ODN2006 resulted in a very potent secretion of IL-8 – a cytokine undesirable in the tumor environment due to its angiogenic properties [61]. The extent of this secretion was considerably lower with MGN1703 and ODN2216 (Fig. 5F).

5.5. Comparison of MGN1703 and CpG-ODN: toxic effects in mice

It is known that PT-based CpG-ODN result in significant toxic effects. Reduced functionality and definition of

Table 2
Activation of cytokine production and cell populations from PBMC by MGN1703.

Cytokine secretion	IP-10	MIP-1b	MCP-1	MIP-1a	IL-8	IL-6	IFN-alpha	IL-12 p70	MIG	TNF-alpha	IFN-gamma
Fold stimulation	++	+	+++	+	+	++	+++	–	–	–	+
Cell activation	CD86+ B cells	CD69+ NK cells	CD69+ NKT cells	CD69+ T cells	CD40+ pDC	HLA-DR+ pDC	CD86+ mDC	HLA-DR+ mDC	CD86+ monocytes	CD169+ monocytes	
Fold stimulation	+	+++	++	++	+	+	++	–	++	+++	

The fold stimulation is calculated using the ratio of mean values of the MGN1703 stimulated cells to the unstimulated cells (control). The scale for stimulation of cytokine secretion is defined as: ≤5-fold = –; >5-fold to ≤25-fold = +; >25-fold to ≤125-fold = ++; >125-fold = +++. For cell activation: ≤1.5-fold = –; >1.5-fold to ≤2.5-fold = +; >2.5-fold to ≤5-fold = ++; >5-fold = +++.

lymphoid organs have been observed in mice studies [73], and these effects may have contributed to the unfavorable risk-benefit profile seen in clinical trials with CpG-ODN, as described above. Studies by Heikenwalder et al. in which the PT-containing ODN1826 was used, led to similar investigations with MGN1703. Injections of MGN1703 did not change liver, spleen or lymph node weight and had only a slight effect on histopathologic changes in liver and spleen tissues. In contrast, ODN1826 led to significant increases in liver and spleen weight, together with liver toxicity (inflammatory reactions and hepatocyte necrosis) and damage to the spleen [63]. This is in line with results from Heikenwalder et al. [73] and provides evidence that PT-modifications in DNA-based TLR-9 agonists should be avoided.

5.6. Preclinical antitumor activity of MGN1703

Preclinical evidence of the antitumor effect of MGN1703 has been reported in several mouse studies. In an SKH1 murine model in which de novo papilloma development was initiated and promoted by topical application of chemicals (7,12-dimethylbenzanthracene and 12-O-tetradecanoylphorbol-13-acetat), MGN1703 significantly reduced the number of mice developing papillomas compared with the saline control group, and the total number of papillomas on all mice was reduced by approximately 50% by MGN1703 [74]. Furthermore, several syngeneic murine tumor models were established: a melanoma model with subcutaneous or i.v. B16 tumor cell challenge, a lung carcinoma model with i.v. 3LL tumor cell challenge and a renal cell carcinoma model with subcutaneous renal tumor cell challenge. MGN1703 was applied at least six times prophylactically or therapeutically and across all models resulted in antitumor effects which were independent of type of tumor cell challenge. In addition, MGN1703 decreased growth of solid tumors and reduced the number of metastatic lung nodules [75]. A cell-based tumor vaccine containing tumor cells modified to express IL-7, granulocyte-macrophage colony stimulating factor (GM-CSF), CD80 and CD154 in a fixed combination with MGN1703 used in a murine model for renal cell carcinoma showed 92% survival compared with 25% survival in the control group. This activity was associated with augmented T cell proliferation (>6-fold), cytotoxic activity against spleen cells (>3-fold), and an increase of CD4+, CD8+ and CD86+ cells at the vaccination site (up to 20-fold) [76].

6. Clinical investigation of MGN1703 in patients with cancer

6.1. Phase I studies in solid tumors: MGN1703 as a vaccine adjuvant

MGN1703 was investigated in two studies at very low doses as an adjuvant to vaccination. The first phase I study

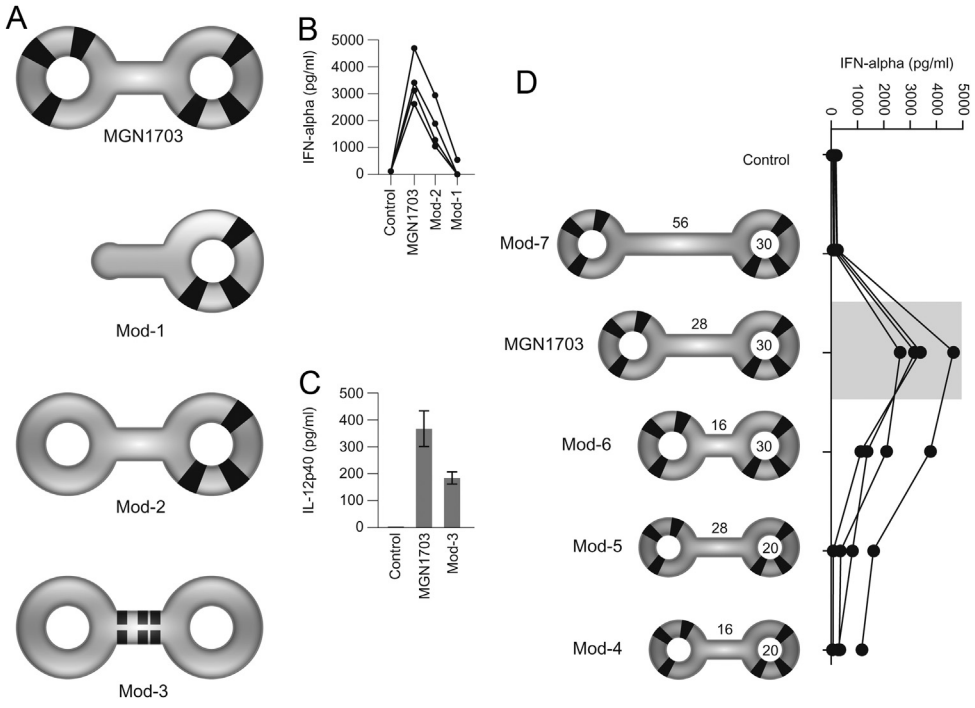

Fig. 4. Influence of structure on function of MGN1703 [63]. (A) Schematic drawing of MGN1703 and various modifications (Mod-1 to -3) with CG motifs in black. (B) Activation of normal donor PBMC to produce IFN-alpha. (C) Activation of IL-12p40 production by murine spleen cells. (D) Schematic draw of MGN1703 and different sized modifications (Mod-4 to -7) with CG motifs in black (left). Numbers of nucleotides is denoted in the loops. Number of base pairs is shown on top of the stem. Activation of normal donor PBMCs to produce IFN-alpha (right). Samples were analyzed by ELISA. Lines connect the samples from the same donor. Reprinted by permission from Mary Ann Liebert, Inc., Schmidt M, et al. Design and structural requirements of the potent and safe TLR-9 agonistic immunomodulator MGN1703. Nucleic Acid Ther 2015, in press, copyright 2015.

of MGN1703 was performed in 10 patients with metastatic colon carcinoma, renal cancer or melanoma [77]. Four subcutaneous vaccinations of autologous tumor cells modified to express IL-7 and GM-CSF were administered in combination with MGN1703. Treatment was associated with encouraging activity: one CR (>24 months), one PR and one mixed response with progression of abdominal metastases but regression of lung metastases. In addition, two patients achieved SD and a further five showed progressive disease (PD) (Table 3). Importantly, AE were not reported in any patient [77]. A second phase I/II study investigated MGN1703 in combination with vaccination and chemotherapy in 17 patients with metastatic CRC [78]. Patients were treated on an alternating schedule of two vaccinations one week apart, followed by a cycle of standard chemotherapy. This pattern was followed for 3 cycles of chemotherapy and after completion, patients continued to receive weekly vaccinations until PD occurred. In this study, patients were randomized to a number of adjuvant-containing vaccinations. MGN1703-containing vaccines were given to 9/17 patients. In detail, five patients (29%) achieved a CR (four receiving an MGN1703 vaccine), one (6%) a PR, five (29%) had SD (four receiving an MGN1703 vaccine) and six patients (36%) showed PD (one receiving an MGN1703 vaccine) (Table 3). Overall, AE reported were few in number and of a mild grade [78]. Vaccinations were generally well tolerated only led to mild and transient side effects such as local skin reactions or short-term body temperature elevation [78].

6.2. *Phase I study in metastatic solid tumors: MGN1703 as a therapeutic drug*

A subsequent open-label phase I dose-finding study has also been completed that evaluated the safety and toxicity of higher doses of MGN1703 in 28 patients with metastatic solid tumors who had failed standard therapy [79]. The study had two parts; a single-dose phase (15 patients) and a multiple-dose phase (24 patients, 11 of which were included from the single-dose phase), with MGN1703 administered subcutaneously at dose levels of 0.25–60 mg in each phase. In the multiple-dose phase, subcutaneous MGN1703 was administered on a twice weekly basis for 6 weeks with the possibility of a further 6 week extension after disease stabilization followed by compassionate use (on request of the investigators). The maximum-tolerated dose was not reached, despite patients receiving the highest dose allowed of 60 mg. The most frequent drug-related AE were generally mild and did not result in any discontinuations. Six out of 15 patients (40%) who received treatment for 6 weeks had SD with the remainder showing PD (Table 3). The six patients with SD continued in the extension phase of whom three (50%) showed SD after 6 further weeks of MGN1703 treatment. A total of four patients were then allowed to continue into

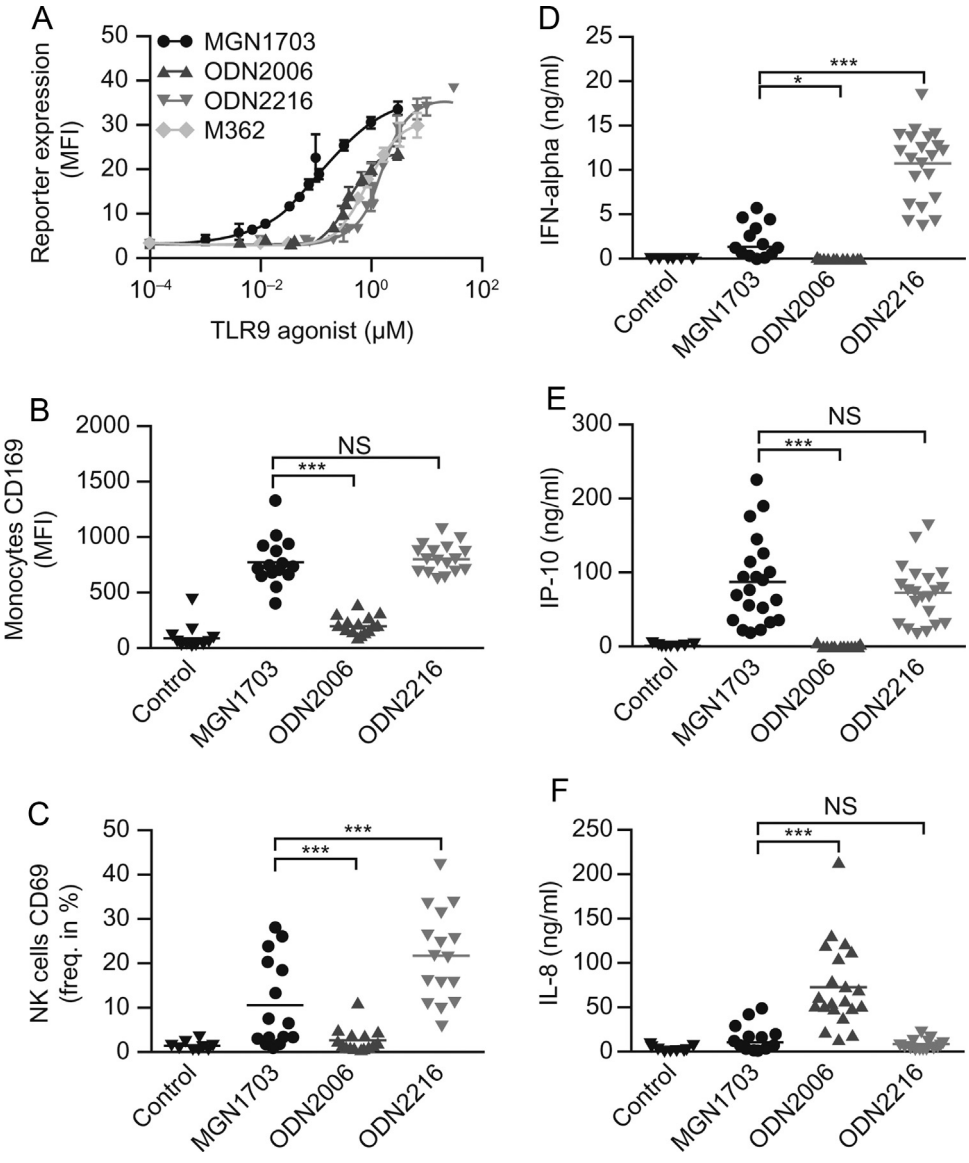

Fig. 5. Immunomodulatory effects of MGN1703 compared with different CpG-ODN [61]. (A) Potency of MGN1703 compared with various CpG-ODN from each class. ELAM41 cells were stimulated with increasing concentrations of MGN1703 or CpG-ODN. (B, C) Effect of MGN1703 and CpG-ODN on activation of immune cells. (D–F) Effect of MGN1703 and CpG-ODN on cytokine secretion. $*P < 0.05$; $***P < 0.001$. IFN, interferon; IP-10, IFN-gamma-induced protein 10; MFI, mean fluorescence intensity; NK, natural killer; NS, not-significant; Control, unstimulated cells. Reprinted by permission from Macmillan Publishers Ltd, Kapp K, et al. Genuine immunomodulation with dSLIM. Mol Ther–Nucl Acids 2014;3:e170, copyright 2014.

compassionate use. Of these, three patients demonstrated prolonged SD with non-small-cell lung cancer (18 months), melanoma (15 months) and CRC (6 months). A fourth patient with CRC achieved almost PR (29.7% of tumor reduction) during this time and continued treatment for 17 months until disease progression [79]. Immunological assessments in this study revealed that treatment with MGN1703 was associated with an increase in the relative number of TLR-9-expressing naïve B cells and a reduction in memory B cells. In addition, a relative increase in the percentage of pDC compared with mDC was reported. Taken together, these early phase studies showed that MGN1703 was well tolerated at doses up to 60 mg and was associated with encouraging activity along with evidence of immune activation in heavily pretreated patients with solid tumors, and CRC in particular [79].

6.3. *Phase II trial of MGN1703 in patients with advanced CRC (IMPACT)*

Following on from the promising phase I trial results, a double-blind phase II trial was performed to evaluate MGN1703 in the maintenance setting for patients who had achieved disease control of metastatic CRC after standard first-line induction therapy [64]. A total of 59 patients were randomized in a 2:1 ratio to receive subcutaneous MGN1703 at a dose of 60 mg or placebo, both given twice weekly until disease progression [64].

Table 3
Clinical responses achieved with MGN1703 in trials to date.

Study	Number of patients receiving MGN1703	Indication	Response for patients receiving MGN1703	Reference
Phase I vaccination ($n=10$)	10	Metastatic colon carcinoma, renal cancer or melanoma	1 CR, 1 PR, 1 mixed response, 2 SD, 5 PD	Wittig et al. (2001) [77]
Phase I/II randomized vaccination followed by chemotherapy ($n=17$)	9	Metastatic CRC	4 CR, 4 SD, 1 PD	Weihrauch et al. (2005) [78]
Phase I monotherapy (n = 28)	24 (28 including single dose group)	Metastatic solid tumors	6 SD, 5 not evaluable[a] (1 patient achieved almost PR during compassionate use), 13 PD	Weihrauch et al. (2014) [79]
Phase II double-blind MGN1703 versus placebo ($n=59$)	43	Metastatic CRC	All patients had at least SD at inclusion (endpoint: PFS); 3 objective responses	Schmoll et al. (2014) [64]

CR, complete response; CRC, colorectal cancer; PD, progressive disease; PR, partial response; SD, stable disease.
[a] Reasons for drop-out of the 5 patients: 2 for regulatory reasons, 2 for protocol deviations, 1 withdrawal of consent.

MGN1703 maintenance treatment was associated with durable PFS in some patients. For the primary endpoint of PFS from the date of randomization to progression on maintenance therapy, hazard ratios (HR) were 0.55 (95% confidence interval [CI], 0.3–1.0; $P=0.04$) and 0.56 (95% CI, 0.29–1.08; $P=0.07$) following investigator and independent review, respectively [64]. The median PFS was 2.8 months (95% CI, 2.8–4.1) with MGN1703 maintenance and 2.6 months (95% CI, 2.5–2.8) with placebo. When measured from the start of induction therapy, PFS statistically significantly improved with MGN1703 compared with placebo following both independent and investigator assessment [64].

Additional subgroup analyses of the primary endpoint suggest that the greatest benefit of MGN1703 may be in patients with a relatively low tumor burden [64]. In this regard, patients with a reduction in tumor size at the end of induction therapy greater than the median (HR 1.015; 95% CI, 1.002–1.029; $P=0.024$), or a CEA concentration within the normal range at the end of induction therapy (HR 0.326; 95% CI, 0.159–0.668; $P=0.002$) had an increased PFS versus patients without these characteristics [64]. However, given the small numbers of patients in this study, these data should be treated cautiously.

At the time of analysis, OS data were not mature. However, the median OS from randomization was 22.6 months (95% CI, 14.9 – not reached) with MGN1703 maintenance and 15.1 months (95% CI, 10.6 – not reached) with placebo (HR 0.63; 95% CI, 0.3–1.5; $P=0.3$) [64]. The median OS from the start of induction was 26.3 months (95% CI, 21.0 – not reached) with MGN1703 and 21.2 months (95% CI, 16.5 – not reached) with placebo (HR 0.65; 95% CI, 0.3–1.6; $P=0.3$).

Three patients in the MGN1703 group had a confirmed objective tumor response (objective response rate 7.0%) during maintenance treatment compared with one placebo-treated patient. Two of the three MGN1703 patients achieved a response only after 9 months of MGN1703 monotherapy, indicating that a carry-over effect from the induction therapy was highly unlikely. At the time of the reported analysis, four patients remained stable having been receiving MGN1703 maintenance for 16–30 months (Table 3) [64].

In a pre-planned analysis of potential immunological biomarkers, the effects of MGN1703 treatment on cellular activation of immunostimulatory cells were apparent with a predominant increase in activated monocytes and pDC [64]. Among patients with high activated NKT cell counts at baseline, MGN1703 led to significantly greater PFS relative to placebo (HR 0.27; 95% CI, 0.14–0.82; $P=0.007$). Although confirmatory data are needed, this suggests that activated NKT cells may be a biomarker for selecting patients most likely to benefit from MGN1703 therapy.

In terms of safety and tolerability, a higher proportion of patients receiving MGN1703 experienced a treatment-related AE relative to placebo (79% versus 38%, respectively). However, treatment-related AE tended to be mainly grade 1 or 2, with one patient discontinuing MGN1703 therapy due to an AE (sensory neuropathy). However, this patient received oxaliplatin chemotherapy before the study. Treatment-related AE of interest because of the MGN1703 mode of action or administration were also mild and included fever ($n=5$), fatigue ($n=4$) and injection-site pruritus ($n=4$) [64]. Only one serious AE was assessed as possibly related to MGN1703 (a patient developed atypical pneumonia with moderate symptoms and was submitted to hospital to confirm diagnosis).

The findings from this phase II study are encouraging, and indicate that maintenance therapy with MGN1703 may improve PFS compared with placebo in advanced CRC, particularly in certain subgroups of patients. In agreement with

earlier studies, MGN1703 was also found to be well tolerated, suggesting that the tolerability profile of this agent is more in line with that of therapeutic vaccines (e.g., sipuleucel-T) rather than the single-stranded TLR-agonists (e.g., PF-3512676). The absence of significant systemic toxicity of MGN1703 may potentially be due its composition of only natural (i.e., non-modified) DNA.

7. Conclusions

The development of TLR-9 agonists in cancer immunotherapy is ongoing with research mainly being directed at a variety of ODN. Despite some initial promise, early generation molecules have not yet been successful in oncology. For example, two phase III trials of the ODN PF-3512676 combined with chemotherapy in non-small cell lung cancer were halted early for futility and the higher incidence of toxicity in the PF-3512676 arm [56,57]. Furthermore, the development of another ODN, IMO-2055, has stalled after the addition of IMO-2055 to cetuximab failed to improve PFS in patients with recurrent or metastatic squamous cell cancer of the head and neck [60].

Compared with CpG-ODN molecules, MGN1703 has a novel structure, resulting in differential stimulation of the immune system compared with the standard classes of CpG-ODN [61]. Additionally, MGN1703 contains only natural DNA components. Clinical trials to date demonstrate that MGN1703 is well tolerated, with mainly grade 1 or grade 2 AE. Promising signs of efficacy have also been reported in a variety of solid tumors (Table 3) [64,77–79]. In particular, encouraging data have been observed in a phase II trial using MGN1703 as maintenance treatment in patients with disease control of advanced CRC. The findings from this study suggest that one avenue for further exploration of this immunotherapy may be in settings with a reduced tumor burden. In particular, MGN1703 may be beneficial to those patients who experience significant tumor shrinkage following induction therapy [64]. IMPALA, a phase III trial of MGN1703 in the first-line/maintenance setting in patients with metastatic CRC, is underway. The IMPALA study will take place in eight European countries and will enroll 540 patients who achieved tumor reduction after receiving first-line chemotherapy with or without biological agents (ClinicalTrials.gov Identifier: NCT02077868). Results from this study, which are expected in late 2017, will offer further insight into the therapeutic potential for MGN1703 as an alternative cancer immunotherapy.

Conflict of interest statement

Burghardt Wittig is an inventor of the DNA-based TLR-9 activation technology. He owns shares in Mologen AG, is an advisor of Mologen AG, and receives funding for the Foundation Institute Molecular Biology and Bioinformatics from Mologen AG.

Manuel Schmidt is an employee of Mologen AG.

Werner Scheithauer and Hans-Joachim Schmoll have no conflicts of interest to declare.

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Acknowledgements

We thank Seren Communications for writing assistance with this manuscript, the funding for which was provided by Mologen AG. We thank Kerstin Kapp for expert review.

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Biography

Burghardt Wittig is a Professor of Biochemistry and Molecular Biology. He is currently Chairman of the Foundation Institute Molecular Biology and Bioinformatics at Freie Universitaet Berlin, Germany. The Institute continues the tradition of his Chair of Molecular Biology and Bioinformatics, which he founded in 1989, when he was appointed full Professor. Prof. Dr. Wittig's basic research fields are the regulation of gene expression through chromatin structural dynamics and the molecular signal transduction of tumor cells. His translational research resulted in DNA constructs for DNA-vaccines against infectious diseases, cell-based gene therapies, and for immunomodulation. Several of these DNA construct-based therapeutic approaches have now entered clinical trials.