

Design and Structural Requirements of the Potent and Safe TLR-9 Agonistic Immunomodulator MGN1703

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Single-stranded oligodeoxynucleotides (ODN), containing nonmethylated cytosine–guanine motifs (CpG ODN), are recognized by the innate immune system as “danger signals.” CpG ODN are efficacious immunomodulators but require phosphorothioate (PT) or other backbone modifications for metabolic stability, which cause toxicities in mice and primates. We therefore designed a covalently closed DNA molecule (dSLIM[®]) where two single-stranded loops containing CG motifs are connected through a double-stranded stem in the absence of any nonnatural DNA component. The most promising immunomodulator, MGN1703, comprises two loops of 30 nucleotides containing three CG motifs each, and a connecting stem of 28 base pairs. MGN1703 stimulates cytokine secretion [interferon (IFN)- α , IFN- γ , interleukin (IL)-12, IL-6, and IL-2] and activates immune cells by increased expression of CD80, CD40, human leukocyte antigen (HLA)-DR and ICAM-1. Efficacy of immunomodulation strictly depends on the descriptive dumbbell shape and size of the molecule. Variations in stem length and loop size lead to reduced potency of the respective members of the dSLIM[®] class. In a representative mouse model, toxicities from injections of high amounts of a CpG ODN-PT and of MGN1703 were evaluated. The CpG ODN-PT group showed severe organ damage, whereas no such or other pathologies were found in the MGN1703 group. Oncological clinical trials of MGN1703 already confirmed our design.

Introduction

OLIGODEOXYNUCLEOTIDES CONTAINING nonmethylated cytosine moieties within cytosine–guanine sequence motifs (CpG ODN) are potent immunomodulators [1,2]. Nonmethylated CG sequence motifs resemble bacterial, certain viral, and also mammalian mitochondrial DNA in terms of this signature [1–5] and are therefore recognized as “danger signals” if such DNA is detected in nonappropriate intracellular environments by the innate immune system. Nonmethylated CG motifs either signal infection as pathogen-associated molecular patterns or are interpreted as the molecular signature of decaying mammalian cells, then termed damage associated molecular patterns. CpG ODN bind and activate Toll-like receptor 9 (TLR-9), which in the human immune system is located in the endoplasmic reticulum of plasmacytoid dendritic cells (pDC) and B cells [6]. TLR-9 activation subsequently triggers a signaling cascade involving MyD88, IRAK, and TRAF-6 leading to the activation of NF- κ B and IRF7 pathways [1,2,7–9]. This results in the production of proinflammatory cytokines and the type 1 interferon response,

leading to the activation and maturation of dendritic cells (Langerhans cells, pDC, myeloid dendritic cells mDC), the proliferation of B cells, and to the proliferation and activation of innate effector cells [e.g., natural killer (NK) cells and invariant NKT cells] among others. Eventually, through the bridging function of mature, activated DC the adaptive immune system responds [10–12].

CpG ODN can be classified into three separate classes with different structural characteristics and differentially enhancing antigen-specific humoral and cellular immune responses: class A are strong inducers of interferon (IFN)- α from pDC but very poor B cell activators and class B are potent stimulators of B cell proliferation with poor induction of pDC IFN- α secretion, while class-C CpG ODN exhibit moderate properties from both class A and class B [13,14].

Being stimulators of the innate and adaptive immune system, CpG ODN have shown promising therapeutic potential as vaccine adjuvants [15–18], as mediators of protective immunity against certain infections [19,20], and as immune therapeutics against cancer [21–23].

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The most extensively studied single-stranded CpG ODN has been the Class B, PF-3512676 (ProMune, CpG-7909 or ODN2006) [24]. Two phase 3 trials of PF-3512676 administered in combination with standard chemotherapy regimens as first-line treatment in advanced non-small-cell lung cancer have not shown improvement in median overall or progression-free survival [25,26]. However, patients receiving PF-3512676 exhibited a higher proportion of adverse events \geq grade 3 in both studies. Due to the lack of significantly improved efficacy and increased toxicity resulting in an unfavorable risk–benefit profile seen with PF-3512676, both trials were terminated early.

Phosphorothioate (PT) modifications of the DNA backbone, usually introduced to enhance stability against degradation in vitro and in vivo [27–29] rendered the resulting ODN-PT much more potent in stimulation of B-cell proliferation than the corresponding ODN with a natural phosphodiester backbone (ODN-PO) [10,30]. However, PT-modifications result in several toxic side effects that may—at least in part—account for the above-mentioned clinical failures: CpG ODN-PT lead to a prolongation of the blood clotting time via inhibition of the intrinsic tenase complex [31–33]. CpG ODN-PT may affect cell signaling by nonspecific binding to various proteins (i.e., transcription factors) [34]. In rhesus monkeys CpG ODN-PT lead to acute toxicities via complement activation [35,36], an effect that also appeared after treatment of normal donor serum with CpG ODN-PT [33]. In rats, CpG ODN-PT were stronger promoters of thrombocytopenia, anemia, or neutropenia than the corresponding CpG ODN-PO [37]; and in mice, the PT-backbone showed a synergistic effect in the induction of arthritis [38]. Most importantly, however, CpG ODN-PT accounted for dramatic destruction and reduced functionality of lymphoid organs in mice [39].

PT or other modifications to protect against exonucleolytic degradation can be avoided if DNA molecules lack open ends like in covalently-closed dumbbell-like structures [40–42]. By this general design principle, we developed a new family of TLR-9 agonists by the name of double stem-loop immunomodulators (dSLIM®). dSLIM are synthesized by ligation of two identical DNA molecules with stable stem-loop structures, resulting in covalently closed molecules of natural DNA (i.e., with a 3', 5'-phosphodiester backbone), which descriptively form a dumbbell-like structure. The immunomodulatory nonmethylated CG motifs are placed in the single-stranded loops, double-stranded stem, or in both.

Here, we present the molecular design path to MGN1703 from other molecules of the dSLIM family. To assay the respective design variants for activation of the TLR-9 pathway, we first employed specific cell lines of different origin as a model system and later confirmed our data in human peripheral blood mononuclear cells (PBMC) from healthy donors. The cell line systems allowed for the detection of subtle differences in TLR-9 activation that would have been lost in PBMC assays due to the individual variability of PBMC from donors. On the other, only in PBMC most aspects of innate and adaptive immune activation by dSLIM molecules could be assessed. Finally, toxicological studies in rodents are reported.

Materials and Methods

TLR-9 agonists for immunomodulation

MGN1703 and other molecules of the dSLIM family were generated as described [43,44]. The endotoxin content of all

nucleotide molecules was determined by an endpoint limulus amoebocyte lysate test to be below 10 EU/mg DNA. If not denoted otherwise, a final concentration of 1 μ M was used for each molecule.

To evaluate the effect of backbone modifications or substitution of CG motifs, we designed several variations (Mod-1 to Mod-9) of the standard MGN1703, a dumbbell-shaped molecule with two single-stranded loops of 30 nucleotides each containing three nonmethylated CG motifs and a 28-bp double-stranded stem. CG motifs were replaced by TG motifs to investigate CG dependence in control molecules (noCpG ODN, noCG-MGN1703).

Cells

RPMI-8226, Jurkat, MOLT-3, U937, NK-92, and YT cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and were cultured as recommended. Cells were stimulated with 1 μ M of respective molecules of the dSLIM family or CpG ODN for 48 hours unless noted otherwise. Human peripheral mononuclear blood cells were isolated from peripheral blood of normal volunteers via Ficoll technique as described previously [45,46].

Flow cytometry

Cells were stimulated with MGN1703 and other molecules of the dSLIM family and flow cytometry was performed as described [43,47]. Briefly, 48 hours after stimulation, cells were washed with phosphate-buffered saline (PBS) and 1×10^6 cells were resuspended and stained with anti-CD80-fluorescein isothiocyanate (FITC), anti-CD86-phycoerythrin (PE), anti-CD40-FITC, anti-CD69-PE, anti-CD54-FITC, anti-human leukocyte antigen DR (HLA-DR)-PE, anti-CD28-FITC, anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4)-PE (all from Becton Dickinson), or anti-human leukocyte antigen ABC (HLA-ABC)-PE (Dako Cytomation) and incubated for 30 minutes. The cells were subsequently washed with PBS [Dulbecco's PBS 0.0095 M (PO₄) w/o calcium and magnesium, Lonza]. The isotypes immunoglobulin G (IgG)1-FITC, IgG2a-FITC, IgG2a-PE, and IgG1-PE (all from Becton Dickinson) were always used as negative controls. Necrotic cells were detected by propidium iodide staining and excluded from analysis. The measurements were carried out using a FACScalibur flow cytometer (Becton Dickinson) and analyzed via CellQuest and WINMDI2.8 Software.

Enzyme-linked immuno assay

Cytokine enzyme-linked immuno assays were performed with supernatants of 10^6 cells/mL that had been cultured for 48 hours according to the manufacturer's instruction [IFN- γ , interleukin (IL)-12p40, IL-6, and IFN- α , R&D Systems].

Stability assay

A stability study according to International Conference on Harmonisation over 36 months at -20°C has been performed for MGN1703. Identity, purity, and content were analyzed via photometric determination, high-performance liquid chromatography (HPLC), and gel electrophoresis. This stability data fulfilled all set specifications.

Toxicology studies in mice

Mice studies were performed as described previously [39]. Briefly, groups consisting of 6-week-old, female C57BL6 mice were administered PBS (control), MGN1703, and ODN1826 intraperitoneally at different doses (in 100 μ L volume) once daily for 7 days. At the end, all mice were sacrificed and organs were weighed immediately. Subsequently, liver and spleen were stored in 4% paraformaldehyde at room temperature. Paraformaldehyde-fixed sections of spleen and liver were stained with hematoxylin/eosin for histological report. Analysis was performed as a double-blind evaluation. An analysis of variance test was used for statistical analysis of organ weights. Mice studies were performed in accredited animal facilities at Dirección General de Agricultura, Comunidad de Madrid (EX-023-USC) following the respective standard operating procedures, directives, and local laws: [European Directives 2003/65/EC/86/609/CEE; Spanish Law RD 223/1988; Humane Endpoints Guidance Document of the Organisation for Economic Co-operation and Development ENV/JM/MONO(2000)7; and Federation of Laboratory Animal Science Associations guidelines].

Results

MGN1703, a member of the dSLIM family

Commonly used PT-modified CpG ODN are known for clinically relevant side effects, such as unspecific protein

binding, prolongation of blood clotting, and acute toxicities [31–39]. As a radical alternative we designed a family of double stem-loop immunomodulatory DNA molecules (dSLIM) with natural phosphodiester (PO) DNA backbones lacking open 3' and 5' ends. Such covalently closed constructs can be drawn as a dumbbell-like shape, where two single-stranded loops are connected through a double-stranded stem. One member of the dSLIM family, MGN1703, consists of a stem of 28 base pairs (bp) and two loops of 30 nucleotides (nt) with identical sequences, containing three nonmethylated CG motifs each (Fig. 1A). The MGN1703 sequence was confirmed by standard sequencing of the primary ODN before the ligation step. Integrity of the covalently closed structure was assayed via exonuclease digestion and subsequent gel electrophoresis or HPLC. MGN1703, when stored at a temperature of -20°C , was stable for a minimum of 36 months (data not shown).

MGN1703 increases expression of immunological surface markers and cytokine secretion

CpG ODN are known to stimulate expression of immunological markers on the cell surface and production of cytokines *in vitro* [1,2]. We investigated the effect of MGN1703 on various cell lines representing components of innate and adaptive immune responses, like B-lineage cells (RPMI-8226), T-lineage cells (Jurkat, MOLT-3), monocytes (U937), and NK cells (NK-92, YT). Analyzing surface expression of

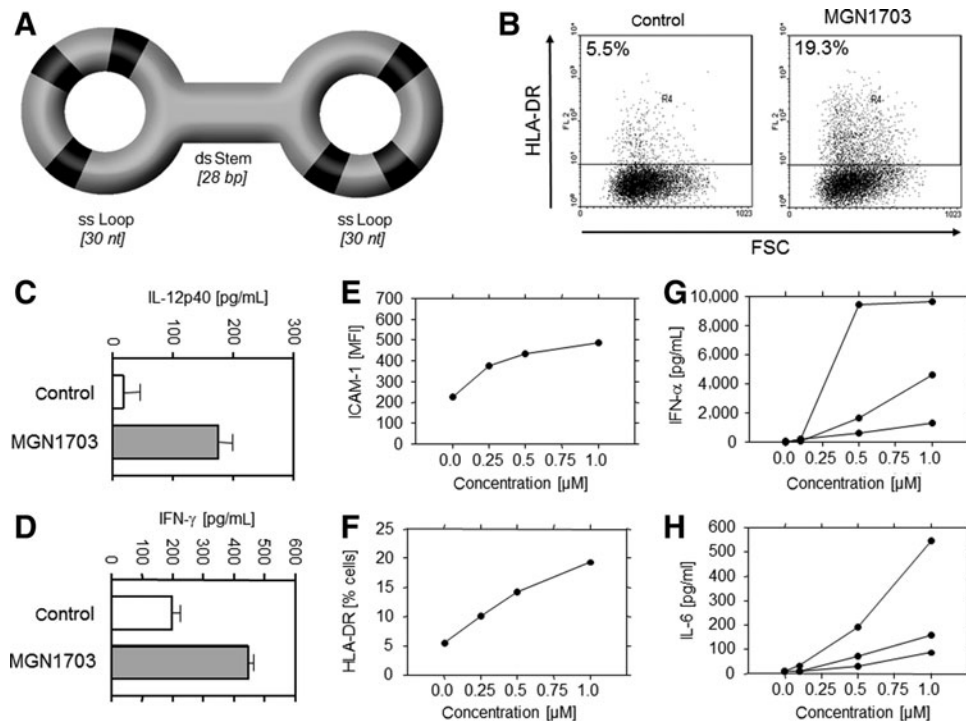


FIG. 1. Immunomodulatory potential of MGN1703. (A) Schematic draw of a member of the double stem loop immunomodulators family (MGN1703) with cytosine–guanine (CG) motifs in black. ds, double strand; ss, single strand (B) Representative flow cytometric analysis of RPMI-8226 cells incubated with or without MGN1703 for 48 hours. FSC, forward scatter. (C) Interleukin (IL)-12p40 enzyme-linked immune assay (ELISA) of supernatant from RPMI-8226 cells. (D) Interferon-gamma (IFN- γ) ELISA of supernatant from natural killer (NK)-92 cells. (E, F) Example of a concentration-dependent stimulation of ICAM-1 and human leukocyte antigen (HLA)-DR surface expression on RPMI-8226 analyzed via flow cytometry. (G, H) Concentration-dependent activation of IFN- α and IL-6 cytokine production from healthy donor peripheral blood mononuclear cell (PBMC) analyzed via ELISA. Each line represents one donor.

TABLE 1. STIMULATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS WITH MGN1703

	<i>IFN-α</i> [pg/mL]	<i>IL-6</i> [pg/mL]	<i>IFN-γ</i> [pg/mL]	<i>IL-12p40</i> [pg/mL]	<i>IL-2</i> [pg/mL]	<i>TNFα</i> [pg/mL]
Control	4	< 25 ^a	< 15 ^a	< 25 ^a	46 \pm 25	16 \pm 1
MGN1703	1712 \pm 342	567 \pm 456	116 \pm 40	117 \pm 25	101 \pm 36	52 \pm 3

Human peripheral blood mononuclear cells were incubated with 1 μ M MGN1703 for 48 hours and cytokine levels analyzed via enzyme-linked immune assay.

Results presented as mean values \pm standard error.

^aBelow the detection level of the enzyme-linked immune assay.

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

immunologically relevant molecules CD80/B7-1, CD86/B7-2, CD40, CD40L/CD154, CD28, CTLA-4/CD152, HLA-DR, ICAM-1/CD54 and CD69 by flow cytometry, we observed no direct effect of MGN1703 on T-lineage cells, NK cells or monocytes. However, B-lineage RPMI-8226 cells showed an increased surface expression of CD80/B7-1, CD40 (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/nat), HLA-DR (Fig. 1B), and ICAM-1/CD54. Regarding cytokine secretion, only a moderate stimulation of IL-12 (p40 subunit) production from RPMI-8226 (Fig. 1C) and IFN- γ secretion from NK-92 cells (Fig. 1D) was found after incubation with MGN1703, confirming that signaling and interaction between different cell types are required to generate the typical downstream effects of TLR-9 activation by MGN1703. In keeping with this, human PBMC, when incubated with MGN1703, induced production of a specific cytokine pattern consisting of T helper cell 1 (T_H1) cytokines IFN- α , IFN- γ , and IL-12p40, and the proinflammatory cytokine IL-

6 (Table 1). All these immunomodulatory effects were clearly dose dependent (Fig. 1E–H).

Immunomodulatory potential of MGN1703 is strictly structure dependent

Some reports described the requirement of nucleic acid secondary structure or the buildup of higher molecular complexes as a prerequisite of immunological efficacy [48,49]. Therefore, we designed variants of stem-loop constructs differing in number and distribution of CG motifs, stem and loop size, and number of loops and evaluated their modulatory potential. Besides MGN1703, these were a single-loop stem construct (Mod-1), a double-loop stem construct with CG motifs in only one loop (Mod-2), and another double-loop stem construct with CG motifs in its stem (Mod-3) (Fig. 2A). All molecules contained the same immunomodulatory sequence, a stretch of at least 20 nucleotides containing 3 CG motifs. Regarding expression of surface

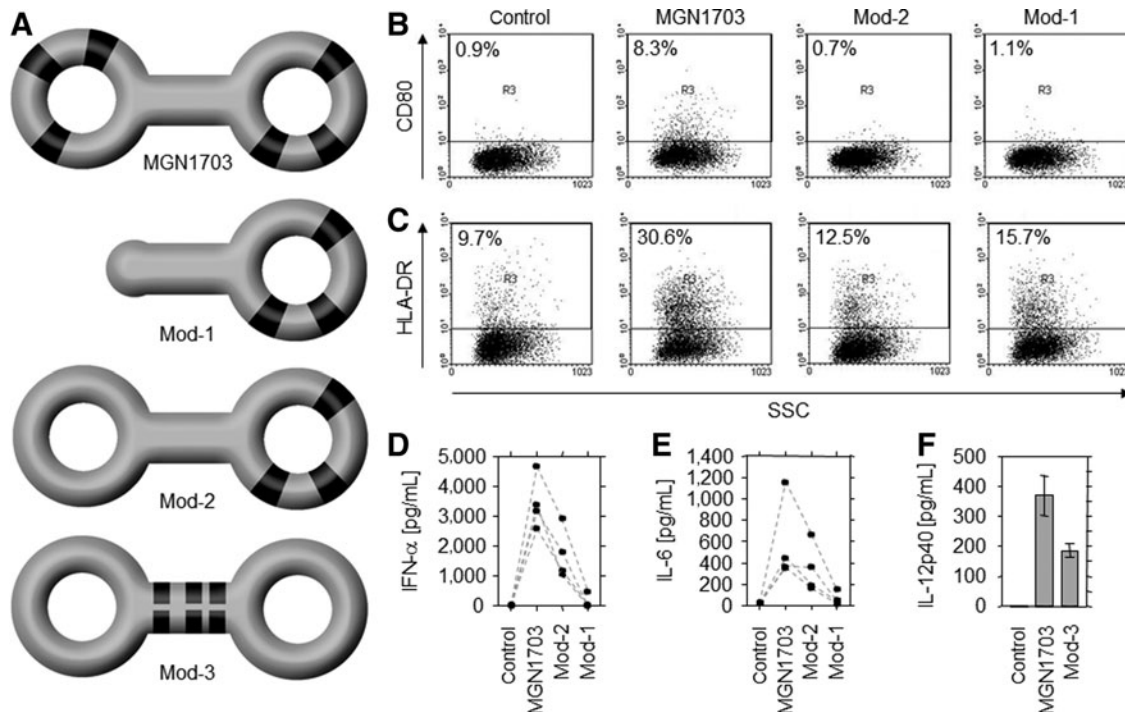


FIG. 2. Influence of structure and CG motif availability on function of MGN1703. (A) Schematic drawing of MGN1703 and modified molecules with CG motifs in black. (B, C) Stimulation of CD80/B7-1 (B) and HLA-DR (C) surface expression on RPMI-8226 cells analyzed via flow cytometry. SSC, side scatter. (D, E) Activation of normal donor PBMC to produce IFN- α (D) and IL-6 (E), which was analyzed via ELISA. Dotted lines connect the samples from one donor. (F) Activation of IL-12p40 production by murine spleen cells as analyzed by ELISA.

molecules CD80/B7-1 and HLA-DR, a change in structure and number of CG motifs reduced the activation potential compared with MGN1703 (Fig. 2B, C).

In keeping with this, MGN1703 also generated the highest stimulation of cytokine production compared with the molecules when IFN- α and IL-6 production from healthy donor PBMC and IL-12p40 from murine spleen cells were analyzed (Fig. 2D–F). To investigate the importance of structure in more detail, we generated further variations of MGN1703 with increased (from 28 bp to 56 bp and 88 bp) or decreased stem size (from 28 bp to 22 bp and 16 bp). Accordingly, we decreased the MGN1703 loop size (from 30 nt to 24 nt and 20 nt) while maintaining the 20-nt immunomodulatory sequence of that encompasses the CG motifs. The resulting molecules Mod-4 to Mod-9 are shown in Figure 3A. Notably, these structure variations of MGN1703 clearly diminished its immunomodulatory potential, as shown by IFN- α , IFN- γ , and IL-6 production from PBMC (Fig. 3B–D). Using even smaller constructs with only one CG motif per loop or two CG motifs in the stem confirmed the necessity of CG motifs in both loops but not in the stem for a high potency (Supplementary Fig. S2). These data clearly demonstrate the

importance of a certain structure, size, CG motif content, and positioning of CG motifs on the efficacy of MGN1703 in both human and murine cells.

MGN1703 does not induce toxicities in rodents

Heikenwalder et al. showed that a CpG ODN-PT resulted in significant toxic effects in mice (i.e., dramatically reduced functionality and definition of lymphoid organs) [39]. To confirm the safety of MGN1703, we used a similar mouse model and successive injections of 2.5 μ g or 60 μ g of DNA-based immunomodulators, comparing MGN1703 consisting of natural DNA entirely with ODN1826 as a CpG ODN with a PT-modified backbone. After seven daily intraperitoneal injections of MGN1703, we did not detect any increase of liver, spleen, or lymph node weight compared with PBS injection, neither with low dose (2.5 μ g) nor high dose (60 μ g) of MGN1703 (Fig. 4A, B).

In contrast, ODN1826 led to significant weight increase of these organs—with the 2.5 μ g dose and even more with 60 μ g of the CpG ODN-PT (Fig. 4A, B). This repeated the experiments with and confirmed the high toxicity of the CpG

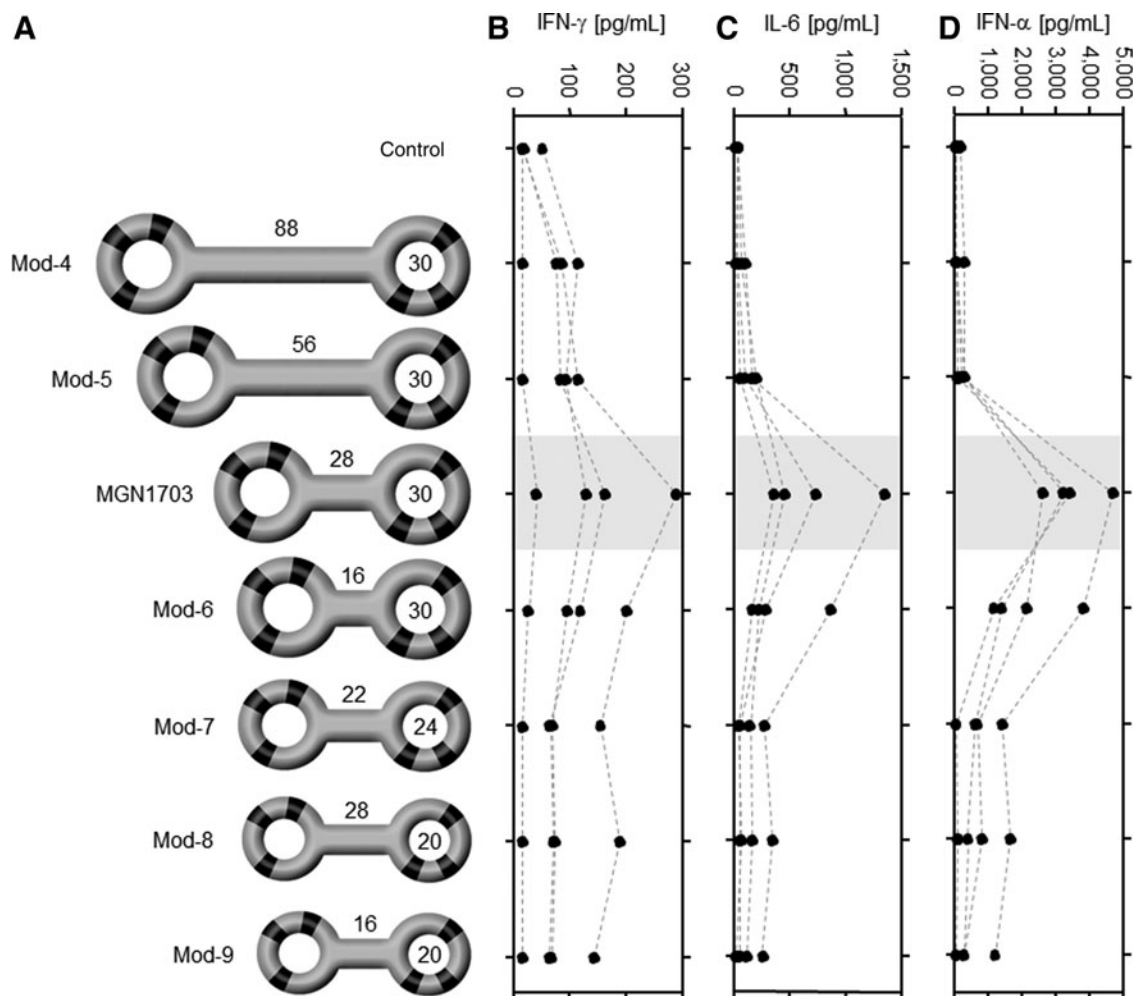


FIG. 3. Influence of size on function of MGN1703. (A) Schematic drawings of MGN1703 and derived molecules with CG motifs shown in *black* (Mod-4 to Mod-9). Numbers of nucleotides are denoted in the loops; numbers of base pairs are shown on top of the stem. (B–D) Activation of healthy donor PBMC to produce IFN- γ (B), IL-6 (C), and IFN- α (D) which was analyzed via ELISA. Dotted lines connect the samples from one donor.

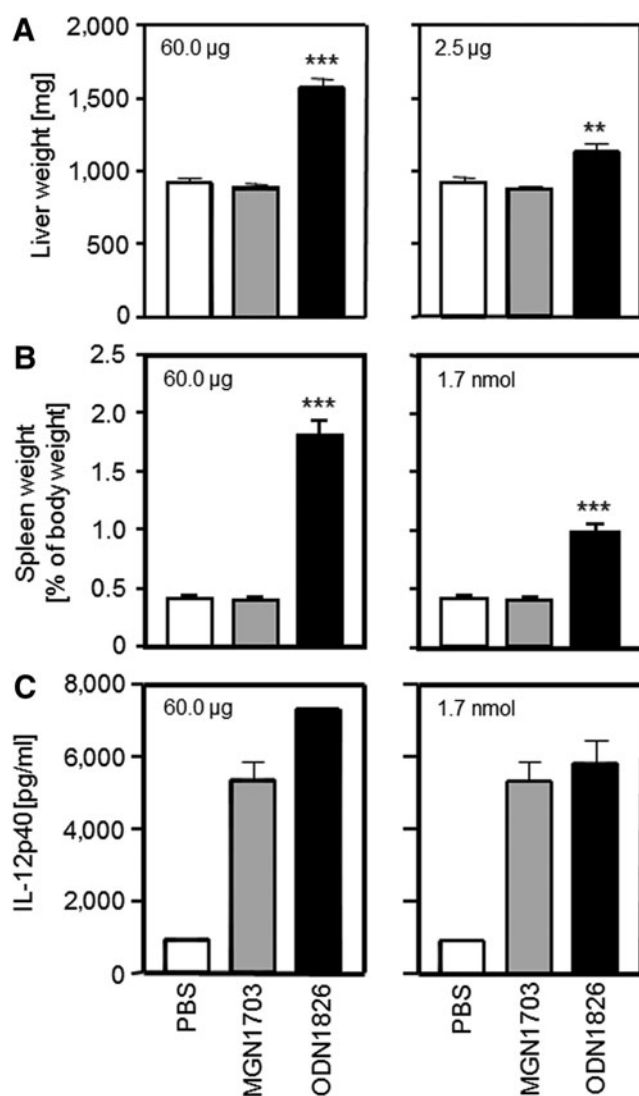


FIG. 4. Effect of MGN1703 and phosphorothioate (PT)-based oligodeoxynucleotide 1826 (ODN1826) on liver and spleen size from mice and cytokine production in vivo. (A) Absolute liver weight after seven daily injections of each 60 µg (left) and 2.5 µg (right) of MGN1703 (gray bars) or ODN1826 (black bars). (B) Relative spleen weight after seven daily injections of equal mass (60 µg, left) and equimolar (1.7 nmol, right) amounts of MGN1703 (gray bars) or ODN1826 (black bars). (C) In vivo serum levels of IL-12p40 after seven daily injections of equal mass (60 µg, left) and equimolar (1.7 nmol, right) amounts of MGN1703 (gray bars) or ODN1826 (black bars). Statistically significant differences to the phosphate-buffered saline (PBS) control are denoted with stars (** $p < 0.01$, *** $p < 0.001$).

ODN described in the paper by Heikenwalder et al. [39]. The absence of toxicity after MGN1703 injections and the drastic toxicity from ODN1826 injections was not due to the difference in molecular weight (MGN1703 > ODN1826) and the resulting higher number of ODN1826 molecules compared with MGN1703 at equal masses of injected molecules. As shown in Figure 4C, the highly significant differences were also observed if equimolar amounts of the respective molecules were injected, though both MGN1703 and

ODN1826 were able to raise IL-12p40 levels in mice sera (Fig. 4C). Histopathological analyses of liver and spleen tissues confirmed the differences as well (Figs. 5, 6). While MGN1703 had only a slight effect on histopathology, ODN1826 resulted in vastly increased liver damage from inflammatory reaction and hepatocyte necrosis (regarding number and size of lesions). Damage of spleen produced similar impressive evidence, such as red pulp hyperplasia with intense extramedullary hematopoiesis and alteration of the white pulp (Table 2). The organ alterations caused by ODN1826 administration are not the equivalent of a normal immune response but do indicate toxic effects on the tissues analyzed.

This led us to evaluate the safety of MGN1703 under the defined conditions of good laboratory practice (GLP). We performed acute toxicity studies in rats by single intravenous injections of 0.8, 2.7, or 10.4 mg/kg of MGN1703 or subcutaneous injections 0.8, 3.1, or 15.4 mg/kg. None of these injections resulted in any toxic effect, neither regarding mortality, clinical signs (i.e., changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, autonomic activity, presence of abnormal movements, etc.) nor significant alterations after macroscopic evaluation of the external surface of the body and necropsy of organs (kidneys, heart, spleen, liver, peritoneum, mesenteric lymph nodes, and thymus) (data not shown).

Discussion

Backbone-modified ODN, especially CpG ODN-PT, have been evaluated in various clinical trials as promising therapeutics or adjuvants in the treatment of allergies, certain infections, and cancer [50,51]. However, they almost all failed in pivotal trials, probably due to a far too narrow therapeutic window. Safety concerns had earlier been raised from results of in vivo mice experiments, where commonly used CpG ODN-PT caused severe side effects such as destruction of lymphoid organs and hepatosplenomegaly [39].

As shown here, we have developed a new family of potent TLR-9 agonist—the dSLIM family of immunomodulators, with its most prominent member being MGN1703—consisting of only natural DNA to avoid toxicities and clinical side effects most likely being caused by the PT modifications, while maintaining specific immune activation properties. Protection against degradation by nucleases is achieved by the covalently closed molecular structure of MGN1703. We have shown that the immunomodulatory effect of MGN1703 is clearly dependent on its structure and the CG motif content as well. This fact is corroborated by experiments, where MGN1703 control molecules with no CG motifs had no relevant immunomodulatory effect (Supplementary Fig. S3).

Among the known side effects of CpG ODN-PT is the prolongation of blood clotting time [31]. As expected, the natural (PO) DNA of MGN1703 did not prolong activated partial thromboplastin time in peripheral blood samples from normal donors, whereas the CpG ODN-PT clearly did (Supplementary Fig. S4).

Selective CpG ODN have been characterized for optimal stimulation of murine, primate, or human cells [52,53]. Unlike PF-3512676 (ProMune, CpG-7909 or ODN2006), which was the TLR-9 agonist most often used in human clinical trials, MGN1703 overcomes such “species specificity,”

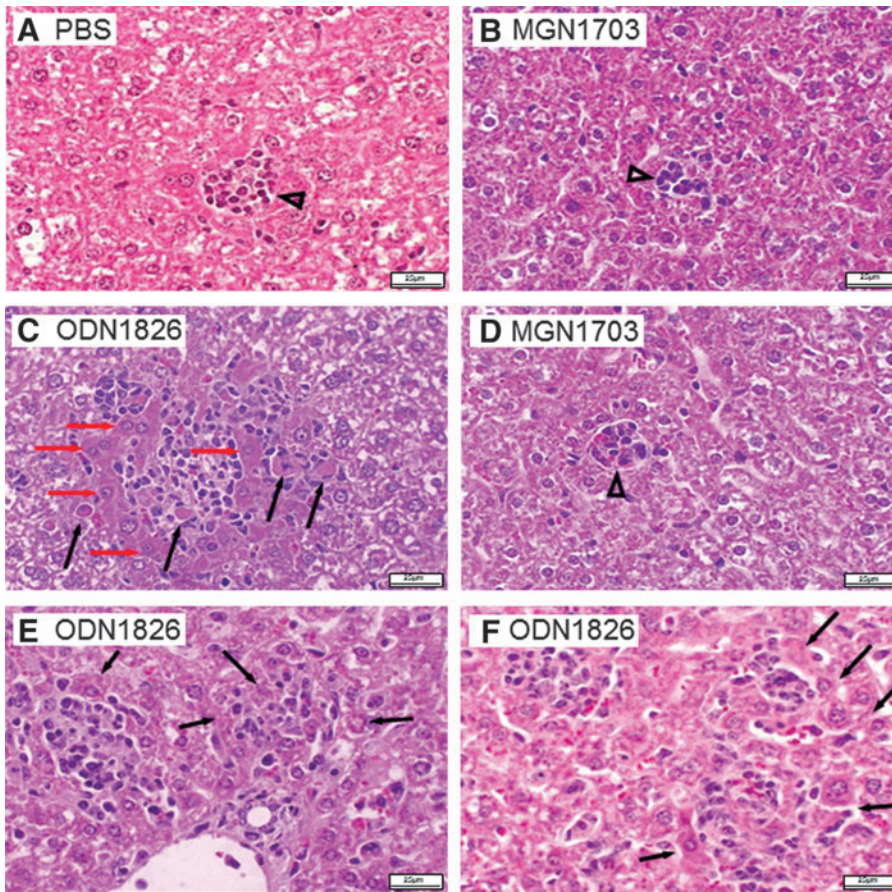


FIG. 5. Histopathology of the liver after seven daily injections of MGN1703 or PT-based ODN1826 into mice. (A) Injection of PBS (control); (B) $7 \times 2.5 \mu\text{g}$ MGN1703; (C) $7 \times 2.5 \mu\text{g}$ ODN1826; (D) $7 \times 60 \mu\text{g}$ MGN1703; (E, F) $7 \times 60 \mu\text{g}$ ODN1826. (A, B, D) Minor inflammatory reaction (*triangles*) predominantly associated to the presence of macrophages and neutrophils. (C) Multiple inflammatory areas with necrotic hepatocytes (*black arrows*) and incipient necrosis (*red arrows*): intense presence of macrophages and some neutrophils. (E, F) Multiple inflammatory areas with incipient necrosis of hepatocytes (*black arrows*): intense eosinophilic response in the cytoplasm, nuclear pyknosis, and karyolysis. Representative pictures are shown.

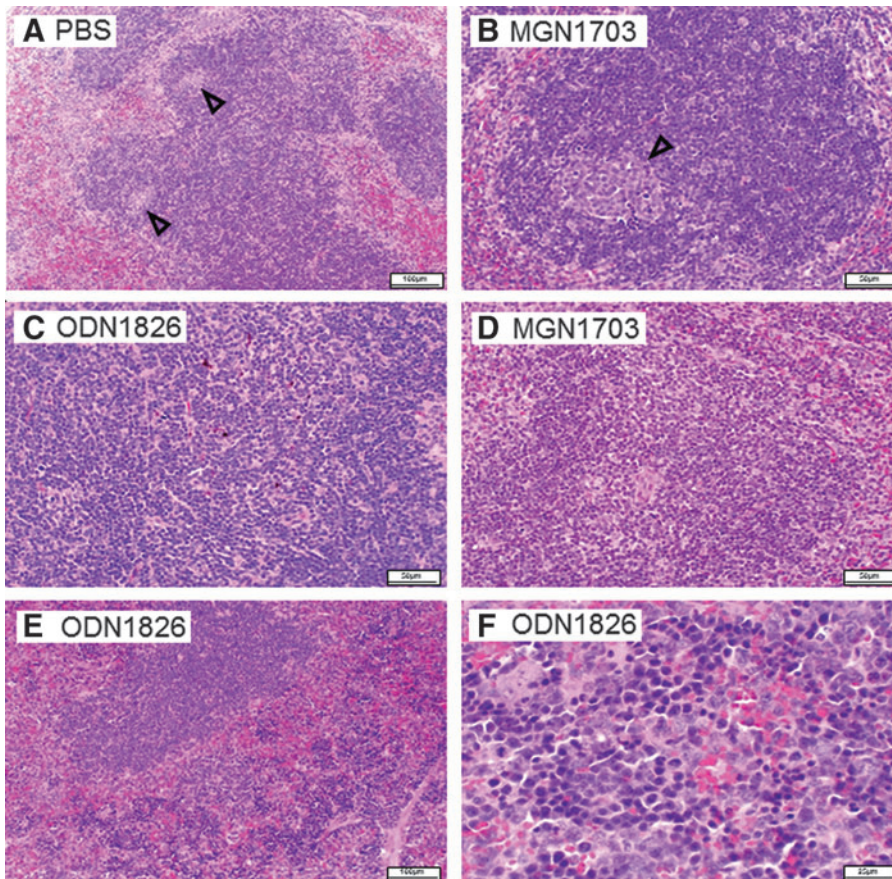


FIG. 6. Histopathology of the spleen after seven daily injections of MGN1703 or PT-based ODN1826 into mice. (A) Injection of PBS (control); (B) $7 \times 2.5 \mu\text{g}$ MGN1703; (C) $7 \times 2.5 \mu\text{g}$ ODN1826; (D) $7 \times 60 \mu\text{g}$ MGN1703; (E, F) $7 \times 60 \mu\text{g}$ ODN1826. (A, B, D-F) White pulp shows a normal structure, including germinal centers (*triangles*). (C) Normal lymphoid cells; no evidence of follicular germinal centers. (E) Red pulp hyperplasia, showing macrophages, myelopoietic, and megakaryocytic cells with extensive histiocytic cells. (F) Red pulp: lymphoplasmocytic and hematopoietic cells with extensive histiocytic cells. Representative pictures are shown.

TABLE 2. HISTOPATHOLOGICAL EVALUATION OF LIVER AND SPLEEN FROM MICE AFTER SEVEN INJECTIONS OF MGN1703 OR PHOSPHOROTHIOATE-BASED ODN1826

<i>Histopathology</i>	<i>Control</i>	<i>MGN1703</i> [60 µg/day]	<i>ODN1826</i> [60 µg/day]	<i>MGN1703</i> [2.5 µg/day]	<i>ODN1826</i> [2.5 µg/day]
Liver damage					
Necrotic hepatocytes	(+)	(+)	+++	(+)	++
Inflammatory reaction	(+)	(+)	+++	(+)	++
Spleen damage					
Uniform lymphoid population	–	(+)	+	–	+
Diffuse lymphoid activation	–	–	+	–	+
White pulp hyperplasia	–	–	–	–	(+)
Red pulp hyperplasia	–	–	+++	–	–

(+)/+/++/+++, degree of changes in relation to ODN1826 [60 µg/day] as group with most severe changes; – not observed.

facilitating the translation of mice experimental results into clinical trials. The efficacy of MGN1703, with its non-modified DNA backbone, in both murine and human cells is perfectly in line with the results of Roberts and colleagues, who have shown that the species specificity of active CpG ODN is restricted to only CpG ODN-PT [54]. A synthetic CpG ODN analogue in which the deoxycytidine was replaced by a bicyclic heterobase also overcomes species specificity. The equivalent molecule but with a PT-modified backbone was rendered toxic and led to spleen enlargement in mice [55].

Secondary structure of MGN1703 as defined by size of double-stranded stem, size and number of loops, and distribution of the CG motifs is crucial for an optimal immunomodulatory potential. A possible explanation why stem-located CG motifs have significantly reduced immunomodulatory efficacy compared with CG motifs positioned in the loop can be derived from the work of Rutz et al. [49]. Binding studies suggest interaction of single-stranded, but not of double-stranded DNA with TLR-9 in a pH-dependent manner. Naturally occurring double-stranded DNA of bacteria is hypothesized to require the acidic conditions in the endosomes for degradation into multiple single-stranded CG motif fragments for subsequent signal transduction by TLR-9. In case of MGN1703, the CG motif located in the single-stranded loop is assessable and may directly interact with TLR-9, whereas in case of the modified molecule Mod-3 degradation of the double-stranded stem containing the CG motifs may be required, thus leading to decreased efficacy in TLR-9 activation.

There are discussions about the interaction of CG motif containing molecules with TLR-9 or with another molecule: On one hand, direct binding of single-stranded non-methylated CpG ODN-PO to TLR-9 in a sequence- and pH-dependent manner has been demonstrated, and a possible DNA binding region within TLR-9 has been identified [49]. However, direct binding of PT ODN to TLR-9 occurred independent of CG motifs and was attributed to nonspecific binding of the PT backbone to proteins. Haas et al. showed that the sugar moieties of the DNA backbone determined recognition by TLR-9 [4]. PO-2'-deoxyribose homopolymers were found to bind and mediate activation of TLR-9. In contrast, PT-modified polymers bound TLR-9 100-fold more strongly, but competitively inhibited ligand-induced TLR-9 stimulation. CG motifs were able to convert this inhibition into strong TLR-9 activation. Accordingly, we hypothesize

that MGN1703 containing only natural PO bonds in its DNA backbone mediates TLR-9 activation via its sugar backbone with CG motifs enhancing its immunomodulatory effects.

The requirement for TLR-9 in MGN1703-induced immunomodulation is corroborated by application of chloroquine, an inhibitor of endosomal acidification – a process necessary for TLR-9 signaling [49,56]. Chloroquine completely abrogated the induction of IFN- γ -inducible protein 10 in MGN1703-stimulated PBMC and strongly diminished the percentage of activated enhanced green fluorescent protein-positive RAW264.7 reporter cells (transfected with human NF- κ B responsive endothelial leukocyte adhesion molecule (ELAM) promoter–green fluorescent protein construct: ELAM 9) (Supplementary Fig. S5) [57]. Another indication for the importance of TLR-9 in the immunomodulation by MGN1703 is the inhibition of the stimulation by ODN with no CG motif (control molecule)—most likely due to competition at the recognition sites of TLR-9. Both, stimulation of surface marker expression as well as cytokine production were clearly reduced when MGN1703 and noCpG ODN were combined for incubation of PBMC or ELAM 9 cells (Supplementary Fig. S3 and Fig. S6). Furthermore, MGN1703 activated enriched pDC but not mDC to produce IFN- α (data not shown); pDC are known to be TLR-9 positive, whereas mDC do not express TLR-9.

TLR-9 has been found to be constitutively expressed as an inactive dimer and to change into an active conformation upon binding of CpG DNA in the endosome so that the cytoplasmic TLR-9 Toll–interleukin 1 receptor signaling domains come in close proximity, enabling signal transduction by recruitment of MyD88 [58]. The specific secondary structure requirements for the optimally active MGN1703 molecule might thus result from a better induction of the conformational change of TLR-9.

On the other hand, a third molecule might hold the two TLR-9 receptors together and could also be involved in TLR-9 activation by holding two TLR-9 molecules together. A possible candidate is heat shock protein 90 (hsp90), which directly binds to CG motifs in CpG ODN-PO [59]. The importance of hsp90 for CpG ODN-PO-mediated signal transduction is also suggested by Okuya et al., who recently showed that hsp90 converted inert self-DNA or mainly CpG ODN-PO into potent triggers of IFN- α secretion [60]. Thus, the importance of secondary structure of MGN1703 might also result from different affinity to hsp90, or yet another unknown molecule involved in CG motif recognition and cellular or endosomal uptake or translocation.

In our mice experiments, the dose of MGN1703 for the toxicological studies was 3 mg/kg and 0.1 mg/kg body weight, adopted from Heikenwalder et al. [39]. Even though the dose of 3 mg/kg is considerably higher than the amount actually used of MGN1703 or CpG ODN in human clinical trials, the dose of 0.1 mg/kg is at the lower end of the general human therapeutic range (0.1–0.6 mg/kg). Still, the low dose of PT-containing ODN1826 showed considerable toxicity in mice, whereas this was not observed with MGN1703. Together with the results of acute toxicity studies from GLP safety, we conclude that an application of MGN1703 should be safe, while exhibiting great immunomodulatory potential.

Consisting only of natural DNA, MGN1703 could better mimic the immunomodulatory effects of bacterial DNA, though avoiding the unwanted side effects of CpG ODN-PT. Thus, the dSLIM family of TLR-9 agonists—with its most prominent member being MGN1703—constitute a new class of efficacious immunomodulatory molecules free from any chemical modifications. In fact, MGN1703 has shown a good safety profile in a phase 1 clinical trial in patients with solid tumors [61]. Furthermore, it is currently being evaluated in clinical trials for the treatment of a variety of cancers. One, a randomized phase 2 trial in patients with metastatic colorectal carcinoma, has recently been completed and shown that MGN1703 maintenance treatment after first-line chemotherapy was well tolerated and appeared to induce durable and prolonged progression free survival and disease control in a subgroup of patients [62].

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Author Disclosure Statement

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References

- Krieg AM. (2002). CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 20:709–760.
- Klinman DM. (2004). Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* 4:249–258.
- Lotze MT, HJ Zeh, A Rubartelli, LJ Spavero, AA Amoscato, NR Washburn N, ME Devera, X Liang, M Tör and T Billar. (2007). The grateful dead: damage-associated molecule pattern molecules and reduction/oxidation regulate immunity. *Immunol Rev* 220:60–81.
- Haas T, J Metzger, F Schmitz, A Heit, T Müller, E Latz and H Wagner. (2008). The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. *Immunity* 28:315–323.
- Kawai T and S Akira. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11:373–384.
- Iwasaki A and R Medzhitov. (2003). Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5:987–995.
- Hemmi H, O Takeuchi, T Kawai, T Kaisho, S Sato, H Sanjo, M Matsumoto, K Hoshino, H Wagner, K Takeda and S Akira. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740–745.
- Häcker H, RM Vabulas, O Takeuchi, K Hoshino, S Akira and H Wagner. (2000). Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J Exp Med* 192:595–600.
- Latz E, A Schoenemeyer, A Visintin, KA Fitzgerald, BG Monks, CF Knetter, E Lien, NJ Nilsen, T Espevik and DT Golenbock. (2004). TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* 5:190–198.
- Krieg AM, AK Yi, S Matson, TJ Waldschmidt, GA Bishop, R Teasdale, GA Koretzky and DM Klinman. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546–549.
- Klinman D, AK Yi, SL Beaucage, J Conover and AM Krieg. (1996). CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci (USA)* 93:2879–2883.
- Sparwasser T, ES Koch, RM Vabulas, K Heeg, GB Lipford, JW Ellwart and H Wagner. (1998). Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol* 28:2045–2054.
- Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. (2006). *Nat Rev Drug Discov* 5:471–484.
- Liu Y, X Luo, C Yang, S Yu and H Xu. (2011). Three CpG oligodeoxynucleotide classes differentially enhance antigen-specific humoral and cellular immune responses in mice. *Vaccine* 29:5778–5784.
- McCluskie MJ and H.L Davis. (1998). CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J Immunol* 161:4463–4466.
- Brazolot Millan CL., R Weeratna, AM Krieg, CA Siegrist and HL Davis. (1998). CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc Natl Acad Sci (USA)* 95:15553–15558.
- Weeratna RD, MJ McCluskie, Y Xu and HL Davis. (2000). CpG DNA induces stronger immune responses with less toxicity than other adjuvants. *Vaccine* 18:1755–1762.
- Verthelyi D, RT Kenney, RA Seder, AA Gam, B Friedag and DM Klinman. (2002). CpG oligodeoxynucleotides as vaccine adjuvants in primates. *J Immunol* 168:1659–1663.
- Krieg AM, L Love-Homan, AK Yi and JT Harty. (1998). CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. *J Immunol* 161:2428–2434.
- Ray NB and AM Krieg. (2003). Oral pretreatment of mice with CpG DNA reduces susceptibility to oral or intraperitoneal challenge with virulent *Listeria monocytogenes*. *Infect Immun* 71:4398–4404.
- Carpentier AF, G Auf and JY Delattre. (2003). CpG-oligonucleotides for cancer immunotherapy: review of the literature and potential applications in malignant glioma. *Front Biosci* 8:115–127.

22. Jahrsdörfer B and GJ Weiner. (2003). CpG oligodeoxynucleotides for immune stimulation in cancer immunotherapy. *Curr Opin Investig Drugs* 4:686–690.
23. Krieg AM. (2004). Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. *Curr Oncol Rep* 6:88–95.
24. Krieg AM. (2012). CpG still rocks! Update on an accidental drug. *Nucleic Acid Ther* 22:77–89.
25. Hirsh V, L Paz-Ares, M Boyer, R Rosell, G Middleton and W Eberhardt. (2011). Randomized phase III trial of paclitaxel/carboplatin with or without PF-3512676 (Toll-like receptor 9 agonist) as first-line treatment for advanced non-small-cell lung cancer. *J Clin Oncol* 29:2667–2674.
26. Manegold C, N van Zandwijk, A Szczesna, P Zatloukal, J Au and M Blasinska-Morawiec. (2012). A phase III randomized study of gemcitabine and cisplatin with or without PF-3512676 (TLR9 agonist) as first-line treatment of advanced non-small-cell lung cancer. *Ann Oncol* 23:72–77.
27. Zamecnik PC, J Goodchild, Y Taguchi and PS Sarin. (1986). Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA. *Proc Natl Acad Sci (USA)* 83:4143–4146.
28. Stein CA, C Subasinghe, K Shinozuka and JS Cohen. (1988). Physicochemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res* 16:3209–3221.
29. Shaw JP, K Kent, J Bird, J Fishback and B Froehler. (1991). Modified deoxyligonucleotides stable to exonuclease degradation in serum. *Nucleic Acids Res* 19:747–750.
30. Krieg A.M, S Matson and E Fisher. (1996). Oligodeoxynucleotide modifications determine the magnitude of B cell stimulation by CpG motifs. *Antisense Nucleic Acid Drug Dev* 6:133–139.
31. Sheehan JP and HC Lan. (1998). Phosphorothioate oligonucleotides inhibit the intrinsic tenase complex. *Blood* 92:1617–1625.
32. Henry SP, W Novotny, J Leeds, C Auletta and DJ Kornbrust. (1997). Inhibition of coagulation by a phosphorothioate oligonucleotide. *Antisense Nucleic Acid Drug Dev* 7:503–510.
33. Shaw DR, PK Rustagi, ER Kandimalla, AN Manning, Z Jiang and S Agrawal. (1997). Effects of synthetic oligonucleotides on human complement and coagulation. *Biochem Pharmacol* 53:1123–1132.
34. Brown DA, SH Kang, S.M Gryaznov, L DeDionisio, O Heidenreich, S Sullivan, X Xu and MI Nerenberg. (1994). Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. *J Biol Chem* 269:26801–26805.
35. Levin AA. (1999). A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim Biophys Acta* 1489:69–84.
36. Henry SP, G Beattie, G Yeh, A Chappel, P Giclas, A Mortari, MA Jagels, DJ Kornbrust and AA Levin. (2002). Complement activation is responsible for acute toxicities in rhesus monkeys treated with a phosphorothioate oligodeoxynucleotide. *Int Immunopharmacol* 2:1657–1666.
37. Agrawal S, Q Zhao, Z Jiang, C Oliver, H Giles, J Heath and D Serota. (1997). Toxicologic effects of an oligodeoxynucleotide phosphorothioate and its analogs following intravenous administration in rats. *Antisense Nucleic Acid Drug Dev* 7:575–584.
38. Bjersing JL, K Eriksson, A Tarkowski and LV Collins. (2004). The arthritogenic and immunostimulatory properties of phosphorothioate oligodeoxynucleotides rely on synergy between the activities of the nuclease-resistant backbone and CpG motifs. *Inflammation* 28:39–51.
39. Heikenwalder M, M Polymenidou, T Junt, C Sigurdson, H Wagner, S Akira, R Zinkernagel and A Aguzzi. (2004). Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat Med* 10:187–192.
40. Erie D, N Sinha, W Olson, R Jones and K Breslauer. (1987). A dumbbell-shaped, double-hairpin structure of DNA: a thermodynamic investigation. *Biochemistry* 26:7150–7159.
41. Chu BC and LE Orgel. (1992). The stability of different forms of double-stranded decoy DNA in serum and nuclear extracts. *Nucleic Acids Res* 20:5857–5858.
42. Clusel D, E Ugarte, N Enjolras, M Vasseur and M. Blumenfeld. (1993). Ex vivo regulation of specific gene expression by nanomolar concentration of double-stranded dumbbell oligonucleotides. *Nucleic Acids Res* 21:3405–3411.
43. Köchling J, SA König-Merediz, R Stripecke, D Buchwald, A Korte, HG Von Einsiedel, F Sack, G Henze, K Seeger, B Wittig and M Schmidt. (2003). Protection of mice against Philadelphia chromosome-positive acute lymphoblastic leukemia by cell-based vaccination using nonviral, minimalistic expression vectors and immunomodulatory oligonucleotides. *Clin Cancer Res* 9:3142–3129.
44. Weihrauch MR, S Ansen, E Jurkiewicz, C Geisen, Z Xia, KS Anderson, E Gracien, M Schmidt, B Wittig, V Diehl, J Wolf, H Bohlen and LM Nadler. (2005). Phase I/II combined chemoimmunotherapy with carcinoembryonic antigen-derived HLA-A2-restricted CAP-1 peptide and irinotecan, 5-fluorouracil, and leucovorin in patients with primary metastatic colorectal cancer. *Clin Cancer Res* 11:5993–6001.
45. Schmidt M, S Nagel, J Proba, C Thiede, M Ritter, JF Waring, F Rosenbauer, D Huhn, B Wittig, I Horak and A Neubauer. (1998). Lack of interferon consensus sequence binding protein (ICSBP) transcripts in human myeloid leukemias. *Blood* 91:22–29.
46. Schmidt M, A Hochhaus, SA König-Merediz, C Brendel, J Proba, G Hoppe, B Wittig, G Ehninger, R Hehlmann and A Neubauer. (2000). Expression of interferon regulatory factor 4 in chronic myeloid leukemia: correlation with response to interferon alfa therapy. *J Clin Oncol* 18:3331–3338.
47. Burchert A, D Cai, LC Hofbauer, MK Samuelsson, EP Slater, J Duyster, M Ritter, A Hochhaus, R Muller, M Eilers, M Schmidt and A Neubauer. (2004). Interferon consensus sequence binding protein (ICSBP; IRF-8) antagonizes BCR/ABL and down-regulates bcl-2. *Blood* 103:3480–3489.
48. Kandimalla ER, L Bhagat, YP Cong, RK Pandey, D Yu, Q Zhao and S Agrawal. (2003). Secondary structures in CpG oligonucleotides affect immunostimulatory activity. *Biochem Biophys Res Commun* 306:948–953.
49. Rutz M, J Metzger, T Gellert, P Lippa, GB Lipford, H Wagner and S Bauer. (2004). Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur J Immunol* 34:2541–2550.
50. Paul S. (2003). Technology evaluation: CpG-7909, Coley. *Curr Opin Mol Ther* 5:553–559.

51. Krieg AM. (2007). Development of TLR9 agonists for cancer therapy. *J Clin Invest* 117:1184–94.
52. Hartmann G, RD Weeratna, ZK Ballas, P Payette, S Blackwell, I Suparto, WL Rasmussen, M Waldschmidt, D Sajuthi, R.H Purcell, HL Davis and AM Krieg. (2000). Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J Immunol* 164:1617–1624.
53. Klinman DM and D Currie. (2003). Hierarchical recognition of CpG motifs expressed by immunostimulatory oligodeoxynucleotides. *Clin Exp Immunol* 133:227–232.
54. Roberts TL, MJ Sweet, DA Hume and KJ Stacey. (2005). Cutting edge: species-specific TLR9-mediated recognition of CpG and non-CpG phosphorothioate-modified oligonucleotides. *J Immunol* 174:605–608.
55. Kandimalla ER, L Bhagat, FG Zhu, D Yu, YP Cong, D Wang, JX Tang, JY Tang, CF Knetter, E Lien and S Agrawal. (2003). A dinucleotide motif in oligonucleotides shows potent immunomodulatory activity and overrides species-specific recognition observed with CpG motif. *Proc Natl Acad Sci (USA)* 100:14303–14308.
56. Steinman RM, IS Mellman, WA Muller and ZA Cohn. (1983). Endocytosis and the recycling of plasma membrane. *J Cell Biol* 96:1–27.
57. Roberts TL, JA Dunn, TD Terry, MP Jennings, DA Hume, MJ Sweet and KJ Stacey. (2005). Differences in macrophage activation by bacterial DNA and CpG-containing oligonucleotides. *J Immunol* 175:3569–3576.
58. Latz E, A Verma, A Visintin, M Gong, CM Sirois, DC Klein, BG Monks, CJ McKnight, MS Lamphier, WP Duprex, T Espevik and DT Golenbock. 2007. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. *Nat Immunol* 8:772–779.
59. Bandholtz L, Y Guo, C Palmberg, K Mattsson, B Ohlsson, A High, J Shabanowitz, DF Hunt, H Jornvall, H Wigzell, B Agerberth and GH. Gudmundsson. (2003). Hsp90 binds CpG oligonucleotides directly: implications for hsp90 as a missing link in CpG signaling and recognition. *Cell Mol Life Sci* 60:422–429.
60. Okuya K, Y Tamura, K Saito, G Kutomi, T Torigoe, H Hirata and N Sato. (2010). Spatiotemporal regulation of heat shock protein 90-chaperoned self-DNA and CpG-oligodeoxynucleotide for type I IFN induction via targeting to static early endosome. *J Immunol* 184:7092–7099.
61. Weihrauch MR, H Richly, MS von Bergwelt-Baildon, HJ Becker, M Schmidt, UT Hacker, A Shimabukuro-Vornhagen, U Holtick, B Nokay, M Schroff, B Wittig and ME Scheulen. (2015). Phase I clinical study of the toll-like receptor 9 agonist MGN1703 in patients with metastatic solid tumours. *Eur J Cancer* 51:146–156.
62. Schmoll HJ, B Wittig, D Arnold, J Riera-Knorrenschild, D Nitsche, H Kroening, F Mayer, J Andel, R Ziebermayr and W Scheithauer. (2014). 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* 140:1615–1624.

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