Recent advances in the development of immunostimulatory oligonucleotides

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Some immune cells recognize distinct molecular structures present in pathogens through specific pattern recognition receptors that are able to distinguish prokaryotic DNA from vertebrate DNA. The detection of invading microbial DNA is based on the recognition of unmethylated deoxycytidyl-deoxyguanosin dinucleotide (CpG) motifs. Synthetic oligonucleotides (ODNs) containing these CpG motifs are able to activate both innate and acquired immune responses through a signaling pathway involving Toll-like receptor 9 (TLR9). Depending on the sequence, length, as well as number and positions of CpG motifs in an ODN, distinct immunostimulatory profiles can be observed. These immunostimulatory profiles can be further modified and fine-tuned by appropriate chemical modifications, leading to preclinical and clinical development of CpG ODNs in cancer, allergy, asthma and infectious diseases.

Keywords Adjuvant, CpG motif, immune stimulation, oligonucleotide, phosphorothioates, plasmacytoid dendritic cell, Th1, Toll-like receptor 9

Abbreviations

bDNA	Bacterial DNA
CFA	Complete Freund's adjuvant
COX-2	Cyclooxygenase-2
CpG	Deoxycytidyl-deoxyguanosin dinucleotide
CTL	Cytotoxic T-cell
DC	Dendritic cell
DNA-PKcs	DNA-dependent protein-kinase catalytic subunit
dsDNA	Double-stranded DNA
ERK	Extracellular receptor kinase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRAK	Interleukin-1R-associated kinase
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
NFκB	Nuclear factor-κB
NK	Natural killer
NO	Nitric oxide
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PGE_2	Prostaglandin E_2
PRR	Pattern recognition receptor
SRA	Scavenger receptor type A
Th1	T-helper type 1
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Introduction

Although it has been known for more than a century that bacterial extracts, such as Coley's toxins, contain potent compounds with the potential to induce regression of established tumors in humans, harnessing the power of the immune system by using synthetic oligonucleotides (oligodeoxynucleotides, or ODNs) to fight and prevent cancer and infectious diseases is a recent discovery. William Coley (1862 to 1936) treated more than 900 patients with bacterial extracts, and achieved > 40% sustained clinical remissions. It was then established that some sequences within bacterial DNA (bDNA) could activate the immune system [1,2]. The missing link was finally discovered by Arthur M Krieg who, while working on antisense ODNs, recognized that some sequence motifs, containing a central deoxycytidyl-deoxyguanosin dinucleotide (CpG) in which the cytosine nucleobase is unmethylated, were responsible for the observed immunostimulatory effects [3••]. bDNA also contains CpG motifs, but at a much higher frequency than in vertebrate DNA and, in contrast to the latter, are not methylated on C(5) of the cytosine base [4]. Therefore, it appears that vertebrates have developed an innate immune system to recognize the pattern of foreign DNA. Thus, bDNA containing CpG motifs or synthetic CpG ODNs are a 'danger signal' to the cell, indicating invasion by a pathogen. In recent years, a variety of different CpG ODNs with various immunostimulatory profiles have been identified. Some of them are currently undergoing preclinical and clinical trials for the potential treatment of cancer, allergic asthma and infectious diseases. In this review, recent studies on the mechanism of action of CpG ODNs will be highlighted; their signaling pathway, the different types of CpG ODNs and their chemical modifications with distinct immuno-stimulatory profiles, as well as their therapeutic and prophylactic applications in certain disease areas, will be presented.

Mechanism of action

Recognition of CpG ODNs by Toll-like receptor 9

Innate immune cells recognize certain molecular structures that are present in pathogens, such as pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors (PRRs). The immune cells are activated upon recognition of PAMPs, and trigger the generation of optimal adaptive immune responses. Such structures were described, and their respective PRRs identified. The Toll-like receptors (TLRs) are a large family of PRRs, consisting of ten different TLR subtypes (TLR1 to TLR10). Recently, TLRs were shown to be essential for the recognition of double-stranded RNA (TLR3), lipopolysaccharide (LPS; TLR4), bacterial flagellin (TLR5), small antiviral compounds (TLR7 and TLR8), and bDNA or CpG ODNs (TLR9) [5••,6-9].

The bacterial genome contains a higher frequency of CpG dinucleotides than vertebrate DNA. These dinucleotides are unmethylated in bDNA, and methylated in vertebrate DNA [10]. Small ODNs with unmethylated CpG dinucleotides are able to perfectly mimic the immunostimulatory activity of bDNA [3••]. Studies using TLR9 knockout mice

demonstrated that this TLR subtype is required to observe the effects mediated by bDNA or CpG ODNs [5••]. An earlier study showed that another protein, the DNA-dependent protein-kinase catalytic subunit (DNA-PKcs), might be involved in the CpG-mediated innate immune activation [11]. However, in a recent study in which mice lacking DNA-PKcs, Ku70 or Ku80 were injected with CpG ODNs, no CpG-mediated effects were observed [12]. These results support the model that CpG signaling is efficiently mediated only via TLR9.

Other studies provide additional evidence that TLR9 plays a critical role in the CpG ODN immune stimulation. Human or murine TLR9 proteins were transfected into non-responsive cells, and CpG ODN responses were reconstituted [13,14,15•]. Immune cells respond very efficiently to 6mer nucleotide motifs with the general sequence purine-purine-CG-pyrimidine-pyrimidine [3••]. To activate human cells, the optimal motif is GTCGTT, whereas GACGTT is the optimal motif for activation of murine cells [3••,16•]. These sequence-specific differences could also be observed with murine or human TLR9 transfectants [14,15•]. Therefore, species-specific CpG ODN motifs are required for efficient initiation of the TLR9-specific signaling pathway.

Only a few types of human immune cells are strongly positive for TLR9 expression. B-cells, as well as plasmacytoid dendritic cells (pDCs), clearly express TLR9 [14,15•,17-20] and can be directly stimulated by CpG ODNs [17,19,21]. Myeloid DCs are negative for TLR9 [19,22], but there is no clear evidence that other cells also express TLR9. In some studies, human monocytes were reported to contain low to background levels of TLR9 mRNA [14,15•,17,20], whereas two other studies could not detect any TLR9 mRNA [18,19]. Although two studies reported a direct response of human monocytes to CpG DNA [21,23], other works demonstrated monocyte activation to be indirect and highly dependent on the presence of pDCs and interferon (IFN)-α [15•,17,18]. In murine cells, however, in addition to B-cells and DCs, monocytes, macrophages and other cells of the myeloid lineage directly respond to CpG ODNs [24,25]. Other leukocytes, such as human or murine αβ- and γδ-T-cells, or natural killer (NK) cells, seem to be indirectly stimulated by CpG ODNs and express, if at all, minimal levels of TLR9 mRNA [17,26-29].

There is increasing evidence that TLR9 acts intracellularly in endosomal locations. Although Chuang *et al* and Takeshita *et al* provided some indication that TLR9 might also be present on the cell surface [13,14], TLR9 and CpG ODNs clearly co-localize intracellularly in lysosomal compartments only [14,30••]. In addition, CpG ODNs require internalization and endosomal maturation to activate TLR9 [3••,30••,31-33]. It is interesting to note that CpG ODNs do not need to be formulated with uptake enhancers to exert their immunostimulatory activity: they work by simple incubation with primary cells or cultured cells lines. In contrast, antisense ODNs usually require special formulation with cationic lipids to facilitate uptake into cells and downregulation of gene expression. When ODNs are applied to cells without the help of cationic lipids, they accumulate to a large extent in vesicles such as endosomes and lysosomes. Since the molecular target of CpG ODNs, namely TLR9, is in the endosomal environment, CpG ODNs are highly effective without the need of cellular uptake enhancers.

Immunostimulatory ODNs are taken up by a sequence-independent mechanism [30••], although poly-guanosine stretches can enhance the uptake of phosphodiester ODNs [34,35]. A variety of cell surface proteins could, in principle, bind CpG ODNs, especially ODNs with phosphorothioate backbone modification, thereby enhancing their unspecific and CpG-independent cellular uptake. Surface immunoglobulins (Igs) on B-cells or scavenger receptor type A (SRA) on myeloid cells could be surface receptors for CpG ODNs [35-38]. However, the SRA is not essential for uptake of CpG ODNs or immune stimulation, as SRA knockout mice were capable of responding to CpG ODNs [39].

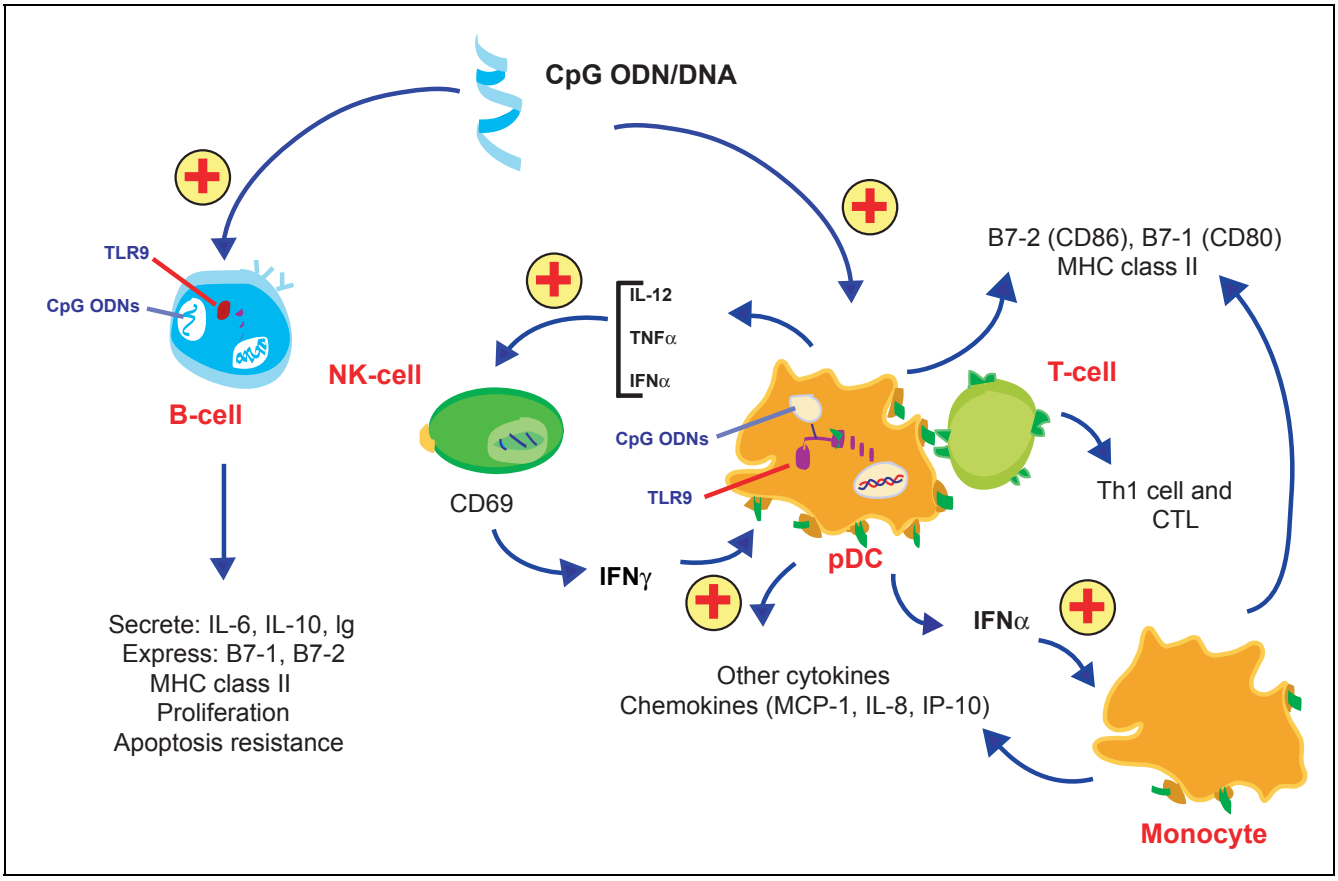
The T-helper type 1 (Th1) cytokine IFNγ upregulates TLR9 mRNA levels, as well as intracellular TLR9 expression [14,30••]. An *et al* [40] demonstrated that LPS upregulated TLR9 gene expression in mouse macrophages. Zarember and Godowski, however, were not able to observe such an effect in human cells [20], and reported that treatment of human peripheral blood mononuclear cell (PBMC) with *Escherichia coli* resulted in downregulation of TLR9. Colony-stimulating factor-1 (CSF-1) suppresses expression of TLR9 and CpG-mediated effects in murine macrophages [41]. TLR9 expression is progressively downmodulated during differentiation induced by CD40 ligands *in vitro* [18]. These results suggest that regulation of cellular TLR9 expression may play an important role in the formation and resolution of infections.

Cell types that respond to stimulation by CpG ODNs

A variety of immune cells are able to respond directly or indirectly to CpG ODNs (Figure 1). Naïve and memory B-cells are readily activated by CpG ODNs [42] and secrete cytokines, such as interleukin (IL)-10, IL-6 or tumor necrosis factor (TNF)-α [43-45]. Human primary malignant B-cells increase the expression of co-stimulatory cell surface receptors or major histocompatibility complex (MHC) molecules upon *in vitro* culture with CpG ODNs [46]. This upregulation might enhance monoclonal antibody (mAb) therapy. Indeed, CpG ODNs were able to enhance the efficacy of mAb in a murine lymphoma model [47].

In vitro, human monocytes only mature into functionally active antigen-presenting DCs through CpG ODN-stimulated pDC cytokine secretion [48]. In murine monocytes and macrophages, CpG ODNs cause direct TLR9-dependent effects [24,25,27]. Two recent studies reported the CpG-dependent upregulation of cyclo-oxygenase-2 (COX-2), expression of nitric oxide (NO) synthase type 2 (NOS2) and production of NO and prostaglandin E_2 (PGE_2) in murine macrophages [49,50]. Inhibition of COX-2 markedly enhanced IFNγ secretion, and CpG ODN-induced PGE_2 downregulated CpG-mediated immune activation. These results indicate that induction of COX-2 in murine macrophages is a control mechanism that regulates excessive Th1 bias. IL-10 secreted by murine

Figure 1. Scheme of CpG DNA-mediated immunostimulatory effects.



Human and murine pDCs and B-cells, as well as murine monocytes and macrophages, express TLR9 and are directly activated by CpG ODNs. These cells upregulate cell surface molecules (eg, CD86 and MHC class II), and secrete cytokines and/or chemokines (eg, IFNα, TNFα, IL-6 and IL-10). Other cells, such as T-cells, NK cells or human monocytes, are indirectly activated by CpG ODNs.

macrophages or B-cells plays an additional negative regulatory role [45,51]. Studies in murine cells or *in vivo* mouse tumor models suggest that IL-10 regulates Th1 immune responses and is an additional factor in the inhibition of antigen-induced IL-5 production [45,52,53].

The main producers of IFNα in human blood are referred to as natural IFNαβ-producing cells, and are identical to CD4+CD123+CD3-CD11c- type 2 DC precursors, or pDCs [54,55]. These cells are unique in their TLR expression and in their ability to directly respond to CpG ODNs by producing cytokines, such as IFNα and IFNβ, or chemokines, such as IL-8 [18,19,21,22,56]. In human PBMC cultures, CpG-induced IFNα secretion is highly dependent on the number of pDCs present [57]. CpG ODNs support survival, activation or maturation of purified human pDCs, and upregulate CCR7, a receptor that drives DC migration to T-cell areas [19,22]. Indeed, CpG ODNs stimulate the migration of DCs: *in vivo* murine Langerhans cells, representing immature DCs in the skin, migrate out of the epidermis upon intradermal CpG injection [58]. CpG ODNs and the CD40 ligand, a signal that can be provided by activated T-cells, synergize the stimulation of pDCs to secrete increased levels of IL-12 p70 and IFNα [19,59,60]. This result, in addition to other studies, demonstrates the strong potential of CpG ODNs to enhance the ability of pDCs to activate allogeneic αβ- or γδ-T-cells [24,29,61]. Recently, a new subpopulation of murine DCs was described, which displays characteristics of human pDC with very strong virus-mediated IFNα secretion, and responds to CpG DNA by increased survival, upregulation of co-stimulatory molecules and production of IL-12 or IL-10 [62,63]. It is tempting to speculate that these cells have a similar role in murine and human CpG-mediated immune activation.

CpG DNA does not seem to directly activate neutrophils. CpG ODNs, unlike LPS, do not induce phenotypic changes or oxidative burst in isolated human neutrophils [64]. In contrast, Bylund *et al* reported a sequence-independent modulation of neutrophil function with phosphorothioate ODN [65]. Nevertheless, *in vivo* studies demonstrated that CpG ODN treatment caused a transient decrease in peripheral blood neutrophils [66••], and led to an enhanced influx of activated neutrophils into the site of a peritoneal infection [67].

A few non-immune cells are stimulated via CpG ODN. Several articles reported the activation of murine cells of the CNS by CpG ODNs. Murine microglia were positive for TLR9 mRNA [27]; moreover, murine microglia and astrocytes could be directly stimulated by CpG ODNs to secrete cytokines and chemokines, or to produce NO

[27,68,69]. Murine microglia are resident macrophages of the brain that initiate an immune response upon infection [70]. As murine cells of the myeloid lineage, in contrast to human cells, express TLR9, the question arises of whether human glial cells of the CNS express TLR9. A recent report indicates that human purified microglia express only low to background TLR9 mRNA levels [71]. In this study, human astrocytes and oligodendrocytes were negative for TLR9 mRNA expression. No study is yet available on the stimulation of isolated human microglia by CpG ODNs.

Osteoclast precursors are another cell type originating from the murine hematopoietic precursor cells that give rise to macrophages or DCs; these cells also express TLR9, and are activated by CpG DNA to release TNFα [72].

The TLR9-mediated signaling pathway

The TLR9-mediated signaling pathway has been partially elucidated and, so far, it seems to be similar for all TLRs investigated and for IL-1R. Recruitment of adaptor molecule MyD88, which is essential for TLR9 signaling, is the first event initiated by CpG DNA, or CpG ODNs, in the endosomal compartments [30••,73,74]. This is followed by engagement of IL-1R-associated kinases (IRAKs) and the adapter molecule TNF receptor-activated factor 6 (TRAF6) [75,76]. Recently, IRAK-M was shown to be a negative regulator of TLR stimulation in murine monocytes and macrophages [77]: macrophages lacking IRAK-M showed enhanced cytokine response upon CpG stimulation or bacterial challenge *in vitro* and *in vivo*. Downstream kinases, such as the mitogen-activated protein kinases (MAPKs), extracellular receptor kinase (ERK), p38 and c-Jun NH_2-terminal kinase (JNK), as well as the IκB complex, are activated, which leads to the activation of transcription factors, such as activator protein (AP)-1 and nuclear factor (NF)-κB [16,51]. One report described the involvement of heat shock protein 90 (Hsp90) in the CpG DNA-induced MAPK signaling pathway [78]. Negative feedback on the MAPK pathways was shown to be mediated by ERK in murine macrophages, and by suppressors of cytokine signaling (SOCS) in the murine macrophage cell line J774 [79,80]. In the same cell line, synergy of CpG ODNs and LPS-mediated TLR activation through NFκB was described [81], although another study suggested that synergy in murine macrophages may occur via a post-transcriptional mechanism [82].

Classes of immunostimulatory DNA

DNA from natural sources

In 1984, it was found that the active immunostimulatory component of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) was in fact formed of DNA [1]. Several studies have since investigated the immunostimulatory activity of different bDNA on vertebrate cells [3••,83-85]. bDNA, in contrast to vertebrate DNA, is hypomethylated and this, in addition to a higher frequency of CpG dinucleotides in a specific sequence context of bDNA, causes the stimulatory effects [3••]. In addition to bDNA, DNA from insects (*Drosophila melanogaster*) [85,86], nematodes (*Caenorhabditis elegans*), yeasts (*Schizosaccharomyces pombe*) and mollusks (*Mytilus edulis*) exhibit varying degrees of stimulatory activity, with *E coli* DNA presenting the strongest stimulus [85], whereas vertebrate, fish and plant DNA are non-stimulatory. A recent report used the DNA of two plants, *Brassica Chinensis* L and *Zea mays*, the methylation status of which are incomplete. Efficient stimulation of murine bone-marrow-derived DCs was observed [87]. Vertebrate DNA, in the form of double-stranded DNA (dsDNA) and upon transfection into the cytoplasm of vertebrate cells, induces upregulation of MHC and co-stimulatory molecules on murine antigen-presenting cells, but does not induce cytokine secretion [88]. These studies demonstrate that immune recognition of CpG DNA may have evolved as an effective and specific defense mechanism in vertebrates.

Classes of CpG ODNs

Some sequence motifs strongly enhance immune stimulation [2,3••]. Hartmann and Krieg described the optimal human hexamer motif GTCGTT [16•]. CpG ODNs bearing such a motif induce specific effects on immune cells *in vitro* and *in vivo* [16•,89,90•,91•]. Phosphorothioate modification of these CpG ODNs enhances their activity by ~ 10- to 100-fold [91•,92]. The CpG motif itself, or the number and position of CpG dinucleotides and the spacing between such motifs, can determine the strength of the immune response induced [91•]. This class of CpG ODNs, class 'B' or 'K' [16•,90•], is a very potent Th1 adjuvant: it presents antitumor activity *in vivo* and *in vitro*, and stimulates strong B-cell and NK-cell activation, as well as cytokine secretion (Table 1) [90•,91•,93,94]. The species specificity of CpG motifs was further investigated by studies performed on immune cells of different domesticated species (sheep, goat, horse, pig, cattle and chicken), rabbits or fish [95-98,99•,100]. These studies demonstrated efficient *in vitro* or *in vivo* immune stimulation by CpG DNA and, in most cases, preferential recognition of the GTCGTT motif for all species (except inbred strains of mice, rats and rabbits).

Table 1. Different classes of CpG ODNs with distinct immunostimulatory properties.

Class of CpG ODNs	B-cells	NK-cells	DCs	Type I IFN induction	Examples of clinical applications
A	+	++++	++++	++++	Cancer, viruses
B	++++	+	++++	+	Cancer, viruses, asthma, vaccines
C	+++	+++	++++	+++	Asthma, allergy, cancer, vaccines

The potency of CpG ODNs to stimulate specific immune cells and to induce type I interferons is shown (from +: weak; to ++++: very strong). This table presents selected examples, and does not summarize the entire potential of CpG ODNs.

Dramatically different profiles of immune activation can be induced, due to differences in ODN sequence or backbone modification independent of the CpG dinucleotide. The thymidine content of a CpG-free phosphorothioate ODN can greatly contribute to immunostimulation [36,92,101]. In contrast, phosphorothioate ODNs rich in other nucleotides (guanosine, cytosine or adenosine) or phosphodiester CpG-

free ODNs are not stimulatory. In addition to the base-content and the backbone chemistry, the length of a phosphorothioate ODN appears to be directly related to the magnitude of its stimulatory effects [101]. Methylation of CpG dinucleotides does not always cause abrogation of immunostimulation, specifically as the length of the ODN is increased [56,101]. These results demonstrate that non-CpG ODNs rich in thymidine or ODNs with methylated CpG motifs have length-dependent effects. Such ODNs can induce effects similar to those observed for class B CpG ODNs, but are less potent and efficient in stimulating human immune cells, and can be associated with Th2-biased *in vivo* immunostimulatory effects in some mouse models [94,101].

The highest degrees of NK stimulation and IFNα secretion occured with ODNs in which the 3'- and 5'-ends were phosphorothioate-modified, and the center portion contained a phosphodiester [102,103•]. The cellular uptake of these chimeric ODNs was enhanced by poly-guanosine stretches at both ends. At least four guanosine nucleotides in a stretch (guanosine-quartet) are necessary to support tetrad formation through Hoogsteen hydrogen bonding. Runs of four guanosine residues at both ends of the ODN lead to intermolecular tetrad formation, resulting in high molecular weight aggregates. These tertiary and secondary structures have a role in efficient immunostimulation by class A, or guanosine-rich ODNs. These ODNs are termed class A ODNs [103•] because of their strong IFNα induction (they are also sometimes called class D ODNs [90•]); they are especially potent in activating human pDCs to produce high levels of IFNα or IFNβ, and have strong *in vivo* antitumor activity in some mouse models [19,89,103•].

Recently, our research team at Coley Pharmaceutical (Germany) defined a new class of CpG ODNs with intermediate immune effects, compared with class A and B ODNs. This new class of CpG ODNs, termed class C, strongly stimulates B-cells (similar to, or better than class B) and type I IFNs and NK-cell activation (slightly weaker than class A). In contrast to class A, which is chimeric and contains poly-guanosine stretches, class C presents no poly-guanosine stretches. Due to their uniform modification by phosphorothioates, class C CpG ODNs are reasonably nuclease-resistant. Furthermore, they strongly stimulate human TLR9-positive cells, pDCs and B-cells, and represent new candidate CpG ODNs for potential applications in cancer therapy and infectious diseases [Krieg AK, Schetter C, Davis H, Vollmer J, unpublished results].

Poly-guanosine stretches provide ODNs with defined additional effects. By enhancing the uptake of CpG ODNs, they improve the immunostimulatory activity of phosphodiester CpG ODNs [34,35,104,105]. ODNs with poly-guanosine runs can co-stimulate T-cells; they can also inhibit the proliferation of prostate cancer cells and induce their apoptosis independently of CpG motifs [28,106].

Methylated calf-thymus DNA or CpG-free phosphorothioate ODNs can also mediate the inhibition of immune activation by bacterial CpG DNA and CpG ODNs [107-109]. The blockage of CpG-mediated immunostimulation by DNA from serotype 2 adenovirus was reported, and neutralizing motifs in the viral DNA were defined [110•]. Further studies by Ashman *et al* and Zhao *et al* [111-113] demonstrated that specific inhibitory sequence motifs, such as GCGGG, were able to block B-cell activation, cytokine secretion and CpG-mediated signaling events. Ashman *et al* suggested that this inhibitory effect was not due to uptake competition with stimulatory CpG ODNs, and that inhibitory ODNs might target an event upstream of CpG-mediated inhibitory kinase (IKK) activation [112]. The suppressive effect of such ODNs is specific to CpG-induced immune responses, and has no effect on LPS-induced activation [111,114]. Suppressive ODNs reduced the manifestation and severity of bacterial DNA-induced arthritis *in vivo* [115]. These results suggest that some pathogens, such as viruses, might have evolved to avoid vertebrate defense mechanisms, through CpG suppression and enrichment of neutralizing DNA sequences. Such DNA sequences could gain importance as therapeutics to treat excessive bDNA-mediated stimulation.

Chemical modifications of CpG ODNs

Chemical modifications of CpG ODNs are used to modulate their stability against nucleases, as well as their cellular uptake characteristics and their immuno-stimulatory profile. Although a plethora of chemical modifications is known from studies on antisense ODNs [116•,117], relatively few of them have so far been applied to CpG ODNs. Since CpG ODNs exert their immuno-stimulatory effect by binding to a protein receptor, while antisense ODNs bind to a complementary target RNA sequence, rules to effectively modify CpG ODNs cannot be directly derived from the literature on antisense ODNs. However, the chemistry underlying both classes of ODNs is basically the same and, therefore, can be used to evaluate CpG ODNs. Most antisense ODNs currently undergoing clinical trials are phosphorothioates, and a great deal of experience has been gained with this structural class of ODNs. Therefore, it is not surprising that phosphorothioate ODNs are currently the CpG derivatives most frequently used *in vitro* and *in vivo*.

Modifications of CpG ODNs include the variation of the phosphodiester linkage or of the heterocyclic nucleobases, as well as alteration of the sugar residue (Figure 2). Finally, some ligands can be conjugated to the CpG ODNs, preferably via their 5'- or 3'-ends.

Modifications of the internucleotide phosphodiester bridge

In DNA, the anionic phosphodiester bond constitutes a naturally occurring internucleotide linkage. In bDNA, the phosphodiester linkage is efficiently recognized by the immune system as long as it contains CpG motifs in the preferred sequence context. As a result of their secondary structure, dsDNAs or larger bDNAs appear to be sufficiently stable to nuclease degradation, while single-stranded phosphodiester-linked ODNs are rapidly degraded *in vivo*. In their pioneering work on CpG ODNs, Krieg *et al* observed immunostimulatory effects with phosphodiester backbone ODNs, whereas phosphorothioate backbone ODNs (**1** and **2**; Figure 3) had more marked effects [3••]. The enhanced effects of phosphorothioate CpG ODNs are

Figure 2. Chemical structure of CpG recognition motif and sites for modification.

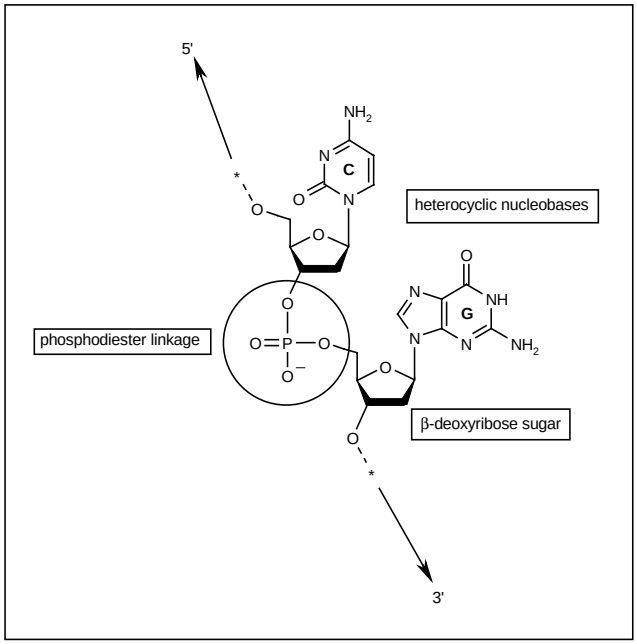
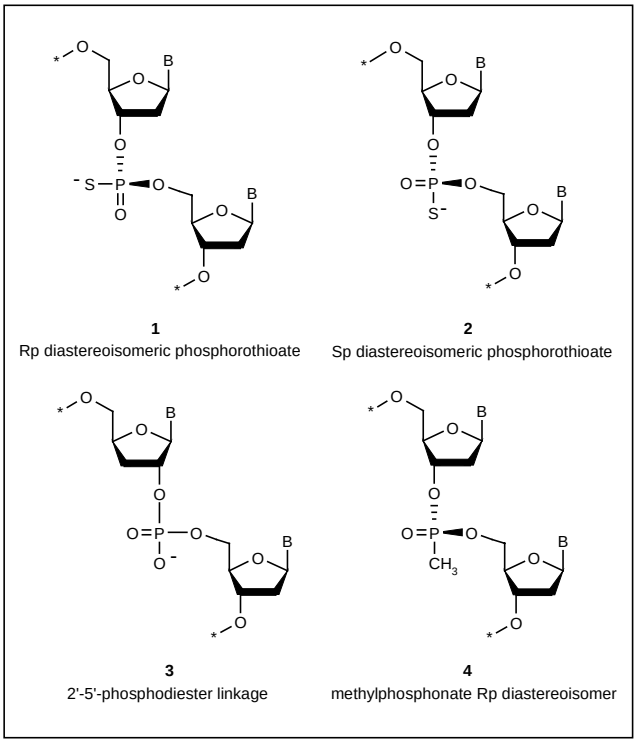


Figure 3. Backbone modifications in CpG ODNs.



B represents a heterocyclic base.

attributed to their increased nuclease stability and their better cellular uptake [118]. However, not every CpG ODN becomes increasingly stimulatory when synthesized with a uniform phosphorothioate modification instead of a phosphodiester or chimeric backbone. Class A CpG ODNs, in particular, can become less active when uniformly phosphorothioated [102]. Both phosphodiester and isoelectronic phosphorothioate linkages seem to be recognized by TLR9. Phosphorothioate ODNs result from synthesis by standard phosphoramidite chemistry as a random mixture of diastereoisomers. However, it was reported that Sp- and Sp-Rp-Sp-chimeric and stereo-random phosphorothioate CpG ODNs evoked higher cell proliferation than the corresponding Rp-phosphorothioate ODNs (**1**; Figure 3) [119]. Although this effect was ascribed to the higher nuclease stability of the Sp-diastereoisomer (**2**; Figure 3), the experimental setup of this study did not allow any conclusion to be drawn as to which diastereoisomer has better affinity for TLR9. It has also been shown that the immunostimulatory properties of CpG ODNs are decreased if the natural 3'-5'-internucleotide linkage is replaced by the unusual 2'-5'-linkage (**3**; Figure 3) [120], which occurs naturally in 2'-5'-linked oligo-riboadenylates. However, when the 2'-5'-linkage was introduced in the 5'-flanking sequence of the CpG motif, slightly increased levels of IL-6 and IL-10, and similar levels of IL-12, were observed in a mouse spleen cell culture assay. The enhancing effects appear to be sequence-dependent, since introduction of the 2'-5'-linkage in the 3'-part to the CpG motif results in decreased secretion of IL-6 and IL-10.

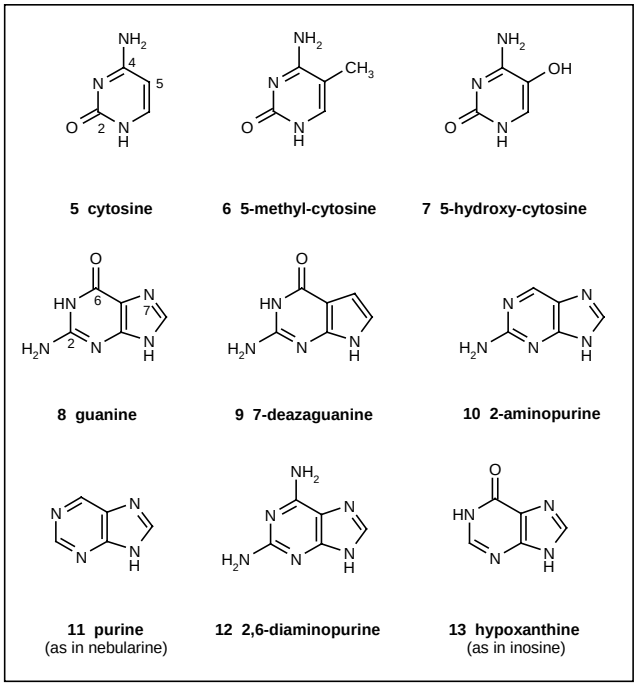
In sharp contrast to the anionic phosphodiester and phosphorothioate ODNs, introduction of a non-charged methylphosphonate linkage at CpG motifs (**4**; Figure 3) results in strongly decreased immunostimulatory activity [121]. However, when the non-ionic methylphosphonate linkage is placed at least four nucleotides away from the CpG motif in the 5'-flanking sequence, cell proliferation and IL-12 secretion in mouse spleen cell culture are increased.

Modifications of the heterocyclic nucleobases

As outlined above, the minimal sequence motif of most immunostimulatory ODNs is a 5'-CpG dinucleotide, in which the cytosine base is not methylated. Simple inversion of this motif to 5'-GpC (3'-CpG) reduces the immunostimulatory properties. Similarly, replacement of cytosine (**5**; Figure 4) by 5-methyl-cytosine (**6**; Figure 4) results in strong reduction or abolishment of immune stimulation [4]. Since this structural difference is the basis for the pattern recognition between bacterial and vertebrate DNA, it is understandable that any modification at the 5-position of cytosine is usually not well tolerated. Thus, most substitutions on C(5) of the cytosine strongly reduce immune stimulation, the only exception being substitution by a hydroxy group (**7**; Figure 4) [122]. Methylation at C(5) of the cytosine seems to affect the activity of short ODNs more [101], whereas methylated CpG ODNs of > 23 nucleotides in length show an *in vitro* activity that is qualitatively similar to that of non-methylated CpG ODNs, though to a much lesser degree. Furthermore, deletion of the 4-amino or 2-oxo functional groups in cytosine, or change to 4-oxo or 2-amino, leads to loss of activity, while alkylation of the 4-amino group is well tolerated [122,123].

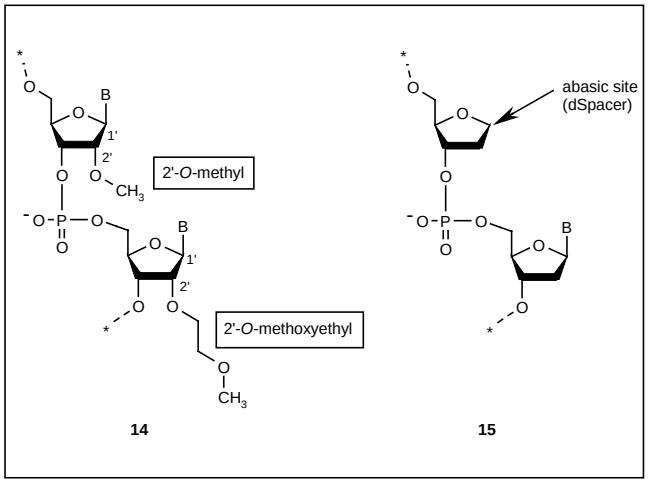
Modification of the guanosine nucleotide of the CpG motif (**8**; Figure 4) in class B CpG ODNs leads to the following ranking in immunostimulatory activity: CpG > Cp(inosine) > Cp(7-deazaguanine) > Cp(nebularine). Deletion of the 2-amino group of deoxyguanosine, corresponding to deoxyinosine (CpI motif, **13**; Figure 4), reduces the ability of B class

Figure 4. Examples of modifications of the heterocyclic nucleobases in CpG ODNs.



Inosine and nebularine nucleosides, respectively, have a hypoxanthine and a purine as nucleobases.

Figure 5. Examples of sugar modifications in CpG ODNs.



In the abasic sugar (dSpacer), the heterocyclic base is replaced by a hydrogen atom. B represents a heterocyclic base.

ODNs to activate NK-cells and to secrete IFNγ, IL-10 and IL-6, but the immunostimulatory potency is still slightly better than that of non-CpG ODNs. Similarly, replacement of the nitrogen at position 7 by a carbon (7-deazaguanine, **9**; Figure 4), deletion of the 6-oxo atom (2-aminopurine nucleoside, **10**; Figure 4), deletion of both 6-oxo and 2-amino (nebularine, **11**; Figure 4) or replacement of 6-oxo by 6-amino (2,6-diaminopurine nucleoside, **12**; Figure 4) in deoxyguanosine all result in decreased induction of cytokines, which, however, are still significantly above the negative controls [Krieg AK, Schetter C, Vollmer J, unpublished results]. In contrast, replacement of two guanosine residues in the guanosine-rich A class ODNs by 7-deazaguanosine, which makes the ODNs incompetent for guanosine-tetrad formation, abolishes the immunostimulatory activity [103•]. Taken together, these results suggest that class B CpG ODNs do not depend on tetrad formation, whereas the ability of forming intermolecular tetrads is an essential structural feature of class A CpG ODNs.

Modifications of the sugar moiety and abasic residues

Recognition of a CpG ODN by its receptor appears to afford a DNA-like sugar conformation rather than an RNA-type sugar conformation at the recognition motif. Hence, CpG ODNs with a 2'-*O*-methyl or a 2'-*O*-methoxyethyl modification at CpG (**14**; Figure 5) [124] show markedly decreased immune stimulation *in vitro* and *in vivo*. If 2'-*O*-alkyl substitutions are introduced at positions distal from the CpG motif, immune stimulation can be either increased or decreased, depending on the sequence, the position of modification, the number of 2'-*O*-alkyl substitutions introduced and the assay system (readout). Deletion of guanosine or cytosine bases through incorporation of an abasic residue, such as dSpacer (**15**; Figure 5), leading to Cp(dSpacer) or (dSpacer)pG, results in strongly decreased but measurable immunostimulatory activity [Krieg AK, Schetter C, Vollmer J, unpublished results]. Immunostimulatory activity depends on the sequence and position of abasic sites. Whereas abasic modification 3' to CpG does not result in changes in activity, abasic modification 5' to CpG can result in increased immunostimulatory activity in mouse spleen cell assays [125]. In another study, the effects of the presence or absence of a nucleoside in the flanking sequences was examined [126]. One or two natural deoxyribonucleosides were replaced at various positions with one or more alkyl groups (two to 12 carbons), branched alkyl groups (glyceryl or aminobutyryl propanediol) or ethyleneglycol- (tri- or hexa-) linkers. A linker substitution at the first two nucleoside positions adjacent to the CpG dinucleotide on the 5'- or the 3'-side abolished the immunostimulatory activity, as determined by *in vitro* mouse spleen cell proliferation and *in vivo* mouse spleen enlargement. When the same substitutions were placed about three to six nucleotides away from the CpG dinucleotide, the immunostimulatory activity was similar or slightly increased, compared with the unmodified parent CpG-DNA. However, in view of the fact that human and mouse cells respond differently to CpG ODNs, it remains to be shown whether results would be similar in human cells.

Therapeutic applications of CpG ODNs

CpG ODNs can activate innate immunity to fight infection

Similar to killed bacteria, which are able to trigger innate immunity, CpG ODN can protect against lethal challenges with a variety of pathogens, such as *Listeria monocytogenes*, malaria, anthrax and Ebola virus. BALB/c mice, which are highly susceptible to *L monocytogenes*, were challenged with ~ 10 LD_{50} of *Listeria* [127]. BALB/c mice were protected against the infectious challenge if they were treated by a single dose (administered intraperitoneally) of a class B CpG ODN 48 h prior to the challenge. Remarkably, the CpG-induced resistance, which appeared to be dependent on IFNγ, persisted for a period of 2 to 4 weeks.

Mice treated with CpG DNA are protected against challenge with *Leishmania major* and malaria. Interestingly, CpG ODNs administered were curative even when given as late as 20 days after lethal *L major* infection [128]. Interestingly, CpG DNA treatment is also effective in protecting mice against challenge with Ebola virus or anthrax [23]. CpG ODNs are generally protective against intracellular pathogens, but not against extracellular bacteria. Using the Friend virus model system, it was demonstrated that enhancement of the immune system by CpG ODNs could lead to dramatic therapeutic effects in retrovirus-induced diseases [129]. When treated 4 days post-infection, recovery increased from 6% in the control group to 74% in CpG ODN-treated animals. CpG ODN treatment promoted a Th1-type cytokine production by splenocytes of Friend virus-infected mice and, furthermore, augmented cytotoxic T-cell (CTL) responses.

CpG ODNs are strong adjuvants

Due to their ability to boost the acquired immune response, CpG ODNs are also predestined for use as vaccine adjuvants. In a study in which 19 different types of adjuvants were compared, a CpG ODN was the most effective for the induction of Th1-like immune responses to tumor antigens [130]. CpG ODNs are also more effective adjuvants than a complete Freund's adjuvant (CFA), without showing the toxic effects of the latter [131]. CpG ODNs are also capable of inducing antigen-specific humoral and cellular immune responses against peptide antigens, viral and bacterial proteins and tumor antigens [132].

Recent findings show that repeated administration of CpG ODNs can enhance the effects of peptide and protein vaccines, leading to potent antitumor responses, likely through the induction of Th1 and DCs, which are essential for optimal CTL responses [133]. Therefore, the immunostimulatory properties of CpG ODNs could be the key to the induction of a consistent long-term immunity to tumor-associated antigens, when using peptides or proteins as T-cell-inducing vaccines.

However, some CpG ODNs that efficiently stimulate human PBMCs are only weakly active in mice, and vice versa. Therefore, an alternative animal model has been developed to monitor the activity and safety of 'human' CpG ODNs *in vivo* [134]. Rhesus macaques recognize and respond to the same CpG motifs that trigger human immune cells. By co-administering CpG ODNs with heat-killed *Leishmania* vaccine to macaques, Verthelyi *et al* obtained significantly increased protection against cutaneous *Leishmania* infection [134]. Furthermore, CpG ODNs are effective as vaccine adjuvants against hepatitis B virus (HBV), which infects only humans and great apes and seems to exist among wild chimpanzees and orangutans [135•]. Since prophylactic vaccination of orangutans with an HBV vaccine proved ineffective, CpG was added as adjuvant to overcome hyporesponsiveness to the HBV vaccine. Addition of CpG DNA to HBV vaccine greatly increased seroconversion rate and antibody titers against hepatitis B surface antigen.

Class B CpG ODNs give potent antibody and CTL responses in primates, humans and rodents. In contrast, class A CpG ODNs show weaker antibody responses but give stronger CTL responses. Guanosine-rich class A CpG ODNs with a chimeric phosphodiester/phosphorothioate backbone, which do not induce lymphadenopathy or prolong cytokine production after local administration, were proposed as an improved adjuvant in vaccination, as they do not lead to long-lasting undesired effects [34].

Since most infectious diseases are transmitted through the mucosal surfaces of the gastrointestinal, genitourinary and respiratory tracts, mucosal immunization may be the most effective way of inducing mucosal immune responses [136•]. In mice, CpG ODNs are effective as mucosal adjuvant with a number of antigens, such as the hepatitis surface antigen [137]. Mucosal delivery, in particular by oral and intranasal routes, would be suitable for mass immunization, and the development of mucosal vaccines using CpG ODNs as adjuvant should be expected in the near future.

Interim results of an ongoing phase I/II safety and efficacy study in normal volunteers using a combination of the CpG ODN CpG-7909 (Coley Pharmaceutical Group) and Engerix B, an HBV vaccine, suggest that CpG-7909 can strongly boost response rates of the HBV vaccine, while increasing antibody titers. Seroprotective antibody levels are reached at 2 weeks in most subjects given the combination, compared with ~ 6 weeks in subjects given the vaccine alone.

CpG ODNs in cancer immunotherapy

Monotherapy

In the treatment of cancer, CpG ODNs can be used either as monotherapy or in combination with mAb or cancer vaccines. Ballas *et al* reported that local or systemic monotherapy with class A CpG ODNs protected 80% of syngeneic C57 BL/6 mice from a lethal challenge of B16 melanoma [89]. However, treatment with a class B CpG ODN was less effective in this model. The fact that SCID mice were also protected against tumor challenge may indicate that neither B- nor T-cells are required. Class A CpG ODNs are potent inducers of NK lytic activity, but have little effect on cytokine secretion or B-cell proliferation. This study demonstrated that selection of optimal CpG ODNs for cancer immunotherapy depends on the careful analysis of the cellular specificities of various CpG motifs and an understanding of the cellular mechanisms responsible for the antitumor activity in a particular tumor.

To evaluate whether CpG ODNs are able to induce rejection of established tumors by monotherapy, Lewis rats were first inoculated intracerebrally with syngeneic CNS-1 glioma cells, and subsequently with CpG ODNs into the tumor bed [138]. Remarkably, 88% ($n = 8$) of the animals treated with a single CpG ODN injection 5 days after tumor inoculation showed long-term survival (> 90 days; $p < 0.002$), whereas all control rats ($n = 14$) died within 23 days. In addition, animals were further protected against a second tumor challenge.

Coley Pharmaceutical Group is currently carrying out a phase I/II study with CpG-7909 in patients with relapsed or refractory non-Hodgkin's lymphoma (NHL). Preliminary results showed that infusions of CpG-7909 were well tolerated. CpG-7909 was administered by 2-h infusions at doses ranging from 0.01 to 0.64 mg/kg, and appeared to

show positive immunostimulatory effects, including increase in median NK activity and trends toward increased numbers of NK-cells. In another study, CpG-7909 is being evaluated in a multicenter phase I/II trial as a monotherapy in patients with stage IV (metastatic) melanoma. Additionally, CpG-7909 has entered a multicenter, open-label phase I/II trial for the treatment of patients with stage IV renal cell carcinoma.

Combination therapy

Since CpG ODNs can orchestrate the immune response to enhance tumor immunity by activating antibody-dependent cell-mediated cytotoxicity, they should improve the therapeutic efficacy of antitumor mAb therapy *in vivo*. Despite the fact that most cancer vaccine studies have so far been carried out with class B CpG ODNs, class A CpG ODNs are also highly effective as vaccine adjuvants. As noted before, the CpG ODN is an extraordinarily effective adjuvant for inducing Th1 responses.

Using the 38C13 B-cell lymphoma model, Weiner *et al* demonstrated that CpG ODNs could function as immune adjuvants in tumor antigen immunization [139]. CpG ODNs were slightly more effective than CFA at inducing an antigen-specific antibody response, but was associated with less toxicity. Interestingly, the CpG ODN induced a higher titer of antigen-specific IgG2a than the CFA, suggesting an enhanced Th1 response. All untreated mice died within 1 month of tumor challenge, whereas mice immunized together with CFA or CpG ODN had 20 or 40% long-term survival, respectively.

In an *in vivo* lymphoma model, a single injection of CpG ODNs dramatically enhanced the antitumor response to antitumor mAb therapy, while CpG ODNs alone showed no effect on the survival of mice inoculated with 38C13 cells [140]. Thus, 90% of mice treated with mAb MS11G6 alone developed a tumor, compared with 20% of mice treated with antibody and CpG ODNs. In contrast, when mice were treated with a control ODN consisting of an identical sequence but with all CpG dinucleotides methylated, these antitumor effects were less pronounced. It is interesting to note that a single dose of the CpG ODN appeared to be as effective as multiple doses of IL-2 to inhibit tumor growth, when combined with antitumor mAb.

Using a mouse model, Warren *et al* demonstrated synergistic effects between CpG ODNs and mAb in the treatment of lymphoma [47]. In this model, CpG ODNs alone had no effect on the survival of animals inoculated with lymphoma. However, when CpG ODNs were administered in combination with a mAb, inhibition of tumor growth was more effective than for mAb alone or mAb plus control ODN. The effects of CpG ODNs were associated with upregulation of the expression of a number of antigens, including CD20.

A phase I study of CpG-7909 in combination with mAb Rituxan (rituximab) in patients with relapsed or refractory NHL is ongoing. CpG-7909 is also being evaluated in a phase I/II clinical trial, in combination with mAb Herceptin (trastuzumab), in patients with refractory metastatic breast cancer.

CpG ODNs in immunotherapy of asthma and allergic diseases

There has been increasing evidence in recent years that Th2-type cytokines IL-4, IL-5, IL-6, IL-10 and IL-13, which are secreted by activated CD4+ T-cells, play a central role in the pathogenesis of allergic diseases, including asthma. In contrast, Th1 cells produce IL-2 and IFNγ. Th1 and Th2 cells interact in a counter-regulatory fashion, as Th2-type cytokines IL-4 and IL-10 promote Th2 development and inhibit Th1 cell development and cytokine production. In contrast, IFNγ inhibits the proliferation of Th2 cells and induces a Th1-type environment [141•]. Consequently, if asthma is caused by a Th2 immune response to inhaled environmental antigens and childhood infections can protect against this, induction of a Th1-like response should prevent the Th2-type immune stimulatory effects causing allergic asthma. The CpG ODN is an extremely effective agent to induce a Th1-like immune response and, therefore, should interfere with the cause of the allergic disease rather than just curing the symptoms, as it is the case with most current therapeutics. Kline *et al* reported that co-administration of a CpG ODN with an antigen in a murine model of asthma efficiently prevented airway eosinophilia, Th2 cytokine induction, IgE production and bronchial hyper-reactivity [142]. Importantly, in a previously sensitized mouse, a CpG ODN prevented allergen-induced airway inflammation, suggesting that exposure to CpG DNA may protect against asthma. In another study, a single dose of a CpG ODN inhibited airway eosinophilia at least as efficiently as daily injections of corticosteroids, the standard treatment for allergic airway disease [143]. Interestingly, only CpG therapy redirected the immune response toward a Th1-like one.

In order to clarify how CpG ODNs can inhibit established Th2 responses, Kitagaki *et al* evaluated the cytokine production from splenocytes from antigen- and alum-immunized mice [144]. These authors found that IFNγ, although playing an important role in the inhibition of antigen-induced IL-5 production by CpG ODNs, was not the sole factor in IL-5 inhibition. CpG ODNs also induced IL-10, which correlates with IL-5 inhibition. These data suggest that CpG ODNs may inhibit established Th2 immune responses through IFNγ and IL-10 secretion, the latter serving to regulate excessive Th1 bias.

Although most of the studies in asthma models have been carried out with class B CpG ODNs, guanosine-rich CpG ODNs are also active in models of allergic asthma. Conjugation of a 3' dG(6)-run to phosphodiester CpG ODNs enhances the production of IFNγ from ovalbumin-specific Th cells, and prevents the development of asthma [104].

Conclusions

The immunostimulatory effects of CpG ODNs, which were originally recognized during studies with antisense ODNs, are exerted at much lower concentration than that required to obtain antisense effects. Typical doses for antisense ODNs *in vivo* are 5 to 10 mg/kg, whereas CPG ODNs are administered in the range of 0.01 mg/kg (adjuvants) to < 1 mg/kg (eg, as anticancer agents). This difference may be explained by the fact that CpG ODNs are recognized by a receptor protein, TLR9, while antisense ODNs by definition recognize their complementary target mRNA sequence. However, when

cells are exposed to an ODN, either CpG or antisense, most of the ODN is trapped in the endosomal and lysosomal compartments. This is where the target of CpG ODNs, TLR9, is located, but antisense ODNs are trapped in this compartment, making it difficult for them to escape to their target mRNA in the cytosol or pre-mRNA in the nucleus. However, many antisense ODNs in clinical trials also contain CpG motifs in their sequence, such as the Bcl-2 antisense [145] or the adenosine A_1 receptor antisense [146], challenging the experimental design of studies with respect to the mechanism of action. However, the development of CpG ODNs has been greatly fostered by the available know-how of both the medicinal chemistry of antisense ODNs and their large-scale production, which include the development of suitable analytical methods for quality control. The fact that the chemical structure of first-generation CpG ODNs (phosphorothioate or phosphodiester-phosphorothioate mixed backbone) is similar to that of developed antisense ODNs has had an extremely positive influence on the speed of development of CpG ODNs as drugs. Therefore, a number of clinical trials with CpG ODNs are currently underway in multiple therapeutic areas, including cancer (eg, NHL, melanoma and breast cancer, either as monotherapy or in combination with mAb), infectious diseases (either as adjuvant for HBV vaccine, or as prophylactic or therapeutic treatment), allergy and asthma. Interim results from phase I/II clinical studies involving ~ 300 individuals suggest that CpG ODN is well tolerated, and that the immune system is activated, as measured by certain markers; finally, antitumor activity has been observed in several cases.

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•• of outstanding interest
• of special interest

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