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Structural requirements for uptake and recognition of CpG oligonucleotides

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Abstract

Recognition of infectious danger by innate immune cells is a fundamental requirement to directly combat infections and to activate the adaptive immune response of T and B cells. Pathogen-associated molecular patterns (PAMPs) play a fundamental role in this process. PAMPs are sensed by pattern recognition receptors, among which the Toll-like receptors (TLRs) play an important role. Within the TLR ligands, bacterial CpG DNA is peculiar. CpG DNA is recognized by TLR9 and harbors the outstanding propensity to induce a milieu that favors activation of Th1-dominated immune responses. This is mainly due to activation of dendritic cells and subpopulations, thereby inducing an intense interferon and IL-12 response. Therefore, CpG DNA has become a promising candidate for constructing new vaccines as well as for induction of immune responses in cancer and allergy. CpG DNA can be synthesized with high purity, defined base composition and various chemical modifications. We aimed to understand the structural requirements for cellular uptake and activation of CpG DNA, which will improve our means to enhance the intrinsic activity of CpG for therapeutic use. We show that sequence modifications can be utilized to enhance cellular uptake, and that chemical substitutions can confer new qualities to synthetic CpG DNA. Additionally, we propose a model of CpG DNA recognition which occurs by sensing partial duplex forms instead of single-stranded DNA. Moreover, we provide evidence that the propensity of CpG DNA to induce high amounts of IL-12 is due to its unique ability to trigger epigenetic modifications of the IL-12p40 promoter, including acetylation and nucleosomal remodeling. Hence, CpG DNA represents a new and promising class of adjuvant for vaccination.

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Introduction

Induction of a long-lasting immunological memory is a central goal in vaccination. Activation of adaptive immune responses of T and B cells is a complex interplay between innate immune cells and naïve cells of the adaptive immune system. According to the

pioneering concept of Charles Janeway Jr., activation of innate immunity is a mandatory prerequisite to launch an adaptive immune response (Medzhitov and Janeway, 1997a). Innate immune cells in turn recognize infectious danger that is brought about by activation of specific receptors that interact with peculiar signatures of non-host cells. These signatures are called pathogen-associated molecular patterns (PAMPs) and are recognized by pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997b). As opposed to clonally distributed receptors of the adaptive immune system, PRRs have

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been selected in phylogeny and thus represent a genetically conserved memory of PAMPs. Accordingly, PAMPs play a pivotal role in initiating an immune response and thus are a key to activate adaptive immune cells and a subsequent immunological memory. Although the generalization of this concept has been challenged recently (Gavin et al., 2006), certain PAMPs and derivatives thereby have been shown to possess strong adjuvant activity (Lipford et al., 1997; Todate et al., 2001; Sieling et al., 2003; Klinman, 2003; Kabelitz and Medzhitov, 2007).

Among the PRRs, the Toll-like receptors (TLRs) are the most prominent and well-characterized group. TLRs are expressed on the surface (TLR 1, 2, 4, 5, 6) or within the endosomal compartment (TLR 3, 7, 8, 9). While the surface-expressed TLRs mainly recognize structural components of pathogens (proteins, lipoproteins, polysaccharides and combinations thereof), the endosomal TLRs are dedicated to recognize polynucleotides (RNA, DNA). It is thought that certain nucleotide sequences or modifications represent the molecular signatures that characterize non-self structures. Accordingly, the endosomal TLRs are responsible for recognition of pathogens which have been phagocytosed or which have invaded the cells (Akira and Hemmi, 2003; Wagner, 2004).

The endosomal expressed TLR9 recognizes the dinucleotide sequence CG (CpG, indicating a phosphodiester bond) embedded in certain conserved motif sequences. CpG sequences are more abundant in prokaryotic than in eukaryotic genomic DNA. Moreover, in eukaryotic DNA the cytosines are methylated, while in prokaryotic DNA methylation occurs only rarely. Thus, unmethylated CpG motifs represent a signature of non-host DNA (PAMP). Not only natural DNA derived from bacteria or viruses but also short (~20 base pairs) synthetic oligonucleotides (ODN) containing CG sequences are sensed by TLR9. Due to the ease of ODN synthesis and manufacturing, CpG-ODN have become a promising candidate for a PAMP-derived adjuvant (Dalpke et al., 2001, 2002b, c).

Our main goal is to analyze the structural and sequence requirements for the efficacy of CpG-ODN as an adjuvant (Akira and Hemmi, 2003; Wagner, 2004; Krieg, 2006).

Recognition of CpG-DNA as a two-step process: uptake into the endosomal compartment is rate limiting for activity

Synthetic CpG-ODN that resemble natural DNA due to their phosphodiester deoxyribose (PO) backbone are quite inefficient. Initially it was thought that this is mainly due to their susceptibility to extra- and intracellular nucleases, which destroy CpG-ODN

rapidly. Therefore, backbone modifications which enhance resistance to nucleases were introduced. Among those, the modification of a thioate deoxyribose backbone (PTO) was most efficient. PTO-ODN were used to describe the first applications of CpG-DNA as an adjuvant (Lipford et al., 1997). However, PTO modification is also disadvantageous. PTO-modified ODN bind to many proteins due to unspecific interactions, and nucleotides with PTO modification which might arise after cleavage in vivo might have carcinogenic potential. Furthermore, PTO-ODN induce immune effects that are independent of CpG motifs and TLR9. In addition, the sulfur atom of PTO modifications leads to a new chiral center, which results in multiple isomers within one ODN preparation.

Rather early it became clear that CpG-ODN stimulation required cellular uptake and could be inhibited by compounds interfering with endosome maturation. Later it was found that TLR9 indeed is expressed in endolysosomal compartments and that CpG-ODN signaling is a two-step process: TLR9 triggering and signal transduction only occur after endosomal uptake.

When we analyzed the uptake of PTO-ODN, we recognized a strong uptake into endosomes which was sequence independent and much more efficient than the uptake of PO-ODN. The uptake of labeled PTO-ODN could be competed by non-labeled PTO-ODN but not by PO-ODN, indicating a specific uptake receptor system. To avoid the constraints of PTO-ODN, we focused in a first series of experiments on development of PO-ODN with an enhanced cellular uptake. We reasoned that addition of unusual nucleotide sequences might hinder nucleases or might induce secondary structures of ODN which could enhance the cellular uptake. Among the modifications tested, addition of poly-G nucleotides was effective. It was known that poly-G nucleotides form secondary structures that consist of multiple ODN (at least quadruple structures). The efficacy of nucleases might be inhibited by these structures, and also uptake might be enhanced. Indeed we observed that poly-G PO-ODN were taken up very efficiently and trigger a CpG-sequence-specific TLR9-dependent immune response. These modifications were effective not only in murine cells but also for human immunostimulatory CpG-PO-ODN (Dalpke et al., 2002a; Zimmermann et al., 2003; Bartz et al., 2004) (for a summary, see Fig. 1).

It was shown for certain proteins that addition of a lipophilic tail enhances their activity. We tried to utilize this observation also for PO-ODN (Schild et al., 1991; BenMohamed et al., 2002). However, addition of lipophilic side chains to the 5' or 3' end of ODN failed to enhance activity. We speculate that these modifications fix ODN to the membrane and thus prevent interaction with TLR9. In contrast, addition of a 5' NH₂-modification enhanced ODN activity, presumably due to an

		cytokine secretion	uptake
PTO	—	-	+++
	— CpG —	+++	+++
	— G —	-	+++
ODN	— CpG — G —	(+)	+++
	—		
	—		
PO	—	-	(+)
	— CpG —	(+)	(+)
	— G —	-	++
ODN	— CpG — G —	++	++
	—		

Fig. 1. Uptake and biological effects of ODN. The results of sequence and backbone modifications on uptake and stimulatory potency are shown schematically. CpG indicates a stimulatory CpG motif, G indicates a poly-G motif.

inhibition of nuclease attack. Accordingly, for our further studies we used poly-G PO-ODN, which we showed to be as efficient as PTO-ODN (Narayanan et al., 2003).

Recognition of CpG-ODN: single-stranded or double-stranded?

Generally it is claimed that synthetic ODN are recognized as single-stranded molecules. However, the natural ligand, bacterial DNA does not occur as a single-stranded molecule – at least after cleavage double-stranded regions are present. To learn about the structural–functional relationships, we entered into a collaboration with Prof. Clemens Richert, Department of Organic Chemistry, University of Karlsruhe. As a first step, we measured the hyperchromicity of various ODN (melting curves). It soon became apparent that hyperchromicity changed dramatically in a temperature region of 15–40 °C, indicating that ODN form complexes at lower temperatures. We take this as an indication that ODN form intermolecular bridges. Detailed analyses at various ODN concentrations finally suggested that ODN form intermolecular but not intramolecular interactions. Thus, at body temperature, ODN at least partly do not occur as single-stranded molecules (Narayanan et al., 2003) but form partial duplex structures. However, the most efficient murine CpG-ODN contain the CG dinucleotide sequence within a self-complementary motif. Moreover, recent results using NMR spectroscopy indeed confirm partial duplex formation of minimal CpG-ODN (He et al., 2007).

By synthesizing specialized ODN with sequences designed to lead to various secondary structures, we were able to formulate a minimal model for the requirements for ODN recognition (Fig. 2). Accordingly, TLR9 recognition requires a partial double-stranded ODN region with a CpG and a single-stranded free 5' end of the ODN (Fig. 2). ODN which are

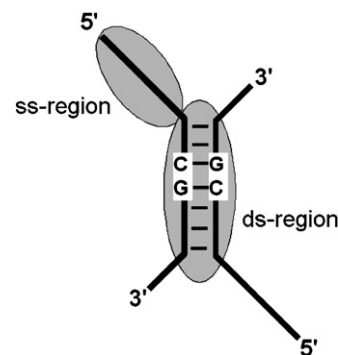


Fig. 2. Working model of CpG-ODN recognition by TLR9. ss – single stranded, ds – double stranded (for details, see text).

constructed to form double strands only were inactive. Analyses further revealed that the single-stranded 5' end has to be without end modification, indicating that this free end plays an important role in interaction with the receptor. Since binding of proteins to DNA is usually restricted to the major groove of the DNA duplex, this model suggests a DNA-binding protein that recognizes CpG motifs in a DNA duplex region. Moreover, during evolution, many protein motifs involved in duplex recognition have evolved (e.g. all transcription factors), yet proteins that recognize specifically single-stranded DNA are rare if existing. Furthermore, duplex recognition is also favored due to thermodynamic reasons. This hypothesis of duplex recognition, which has been substantiated by us in different ways, has enabled us to design multiple new ODN with TLR9 activity (Narayanan et al., 2003; He et al., 2007).

Inhibitory DNA sequences

While PO-ODN with a G-rich end showed enhanced cellular uptake and TLR9-stimulating activity, G-rich PTO ODN failed to stimulate TLR9-expressing cells (Lipford et al., 2000; Dalpke et al., 2002a; Bartz et al., 2004). In analyzing this effect, we noticed that G-rich PTO-ODN, independent of their nucleotide sequence, were able to inhibit the activity of CpG-ODN, irrespective of whether the CpG-ODN were thioate-modified or were of natural phosphodiester structure. At first glance it seemed that inhibition of G-rich PTO-ODN was mainly due to inhibition and/or competition for uptake. Indeed G-rich PTO ODN competed for uptake of other ODN. However, while uptake competition occurred mainly at equimolar concentrations, the inhibitory effect was also seen at lower concentrations (30-fold excess of CpG ODN over G-rich ODN). Moreover, timing experiments revealed that even after 4 h after starting a response with CpG ODN, G-rich ODN were capable of totally suppressing the response. Thus, an interruption of the signaling

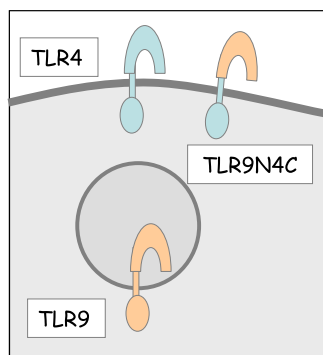


Fig. 3. TLR fusion constructs used (see Barton et al., 2006).

cascade, presumable at the level of TLR9 receptor recognition, explains the activity of G-rich ODN. Furthermore, G-rich ODN failed to inhibit the immune stimulation induced by other TLR ligands and this held true even for other endosomal TLRs, triggered by imiquimod, R848 or polyIC (TLR3, 7, 8). Thus it is unlikely that a general inhibition of endosomal trafficking or uptake is responsible for the inhibition.

To further prove this conclusion, we took advantage of a recently described fusion receptor. When the ectodomain of TLR9 was fused to the transmembrane and intracellular part of TLR4, surface expression of TLR9 was achieved (Barton et al., 2006) (Fig. 3). This chimeric protein is functional since CpG ODN elicits a response that involves the intracellular signal part of TLR4. Using this construct, we were able to show that CpG PTO and CpG PO ODN were active and that both activities could be blocked by G-rich ODN. These results indicate that G-rich ODN directly act as antagonists of CpG-ODN in TLR9 binding. Involvement of downstream signal cascades is unlikely since lipopolysaccharide (LPS)-triggered responses (which utilize the same signal cascades as the TLR9/TLR4 chimeric proteins) were not inhibited. Thus, G-rich ODN could be used as a CpG antagonist to inhibit undesired responses due to TLR9 triggering, as suggested in some autoimmune diseases (Peter et al., unpublished, manuscript submitted).

TLR9-dependent recognition of CpG DNA at the cell surface

The above-mentioned TLR9/TLR4 fusion constructs allowed us to address several questions of CpG-DNA recognition by TLR9 that are unbiased due to alterations in uptake. So far, restrictions caused by differences in uptake of ODN were circumvented by the use of gene transfection chemicals, e.g. DOTAP. However, these agents are toxic and form complexes in undefined cellular compartments. Nevertheless, experiments have shown that CpG-DNA including PO ODN and

endogenous mammalian DNA can cause activation of TLR9. However, at least in the case of mammalian DNA the induced cellular response was minute. When we analyzed various sources of CpG-DNA on the fusion constructs, we found also a weak activity of endogenous mammalian DNA, indicating that TLR9 might recognize endogenous DNA, yet that the natural endosomal localization of TLR9 prevents its physiological relevance. On the other hand, we found that the so-called species specificity of TLR9 recognition breaks down, provided TLR9 is expressed on the surface. CpG-ODN with CpG nucleotide sequences which were normally active only on murine or human cells, respectively, now elicited an immune response that was almost identical (unpublished results). This indicated that certain nucleotide sequence constraints of different species might not be due to direct TLR9 binding but rather due to species-specific alterations in uptake and processing. It is obvious that the formulation of optimal ODN sequences for distinct species will profit from these findings.

Site-directed mutagenesis to reveal TLR9 recognition of CpG DNA

The three-dimensional structure of TLR3 was resolved by X-ray crystallography. TLR3 consists of a horseshoe-like extracellular domain that is formed by an array of leucine-rich repeats (LRRs) (Bell et al., 2003, 2005, 2006). From sequence similarity, a similar structure was deduced for TLR7, TLR8 and TLR9. Interestingly, in these TLRs, a further hinge region can be identified which divides the extracellular domain. Moreover, amino acid insertions within the LRRs and in between the LRRs loom into the concave portion of the domain (Fig. 4). It has been speculated that these loops are responsible for binding of CpG DNA. In order to address this question, we have started to introduce point mutations in these regions by site-directed mutagenesis. So far we have introduced several amino acid substitutions in different loops. Although all constructs were expressed, we observed a complete loss of function. Conservative point mutations were without effect, i.e. these TLR9 constructs recognized CpG-DNA (unpublished results). These results indicate that the loops seem to be essential for binding of CpG-DNA. To obtain deeper insight, we now started to shuffle the looming loops between TLR8 and TLR9 in order to transfer the respective specificities for nucleotide recognition.

Epigenetic regulation of IL-12p40 expression by CpG DNA

When we analyzed the biological validity of several PAMP-derived compounds as a Th1-promoting adjuvant,

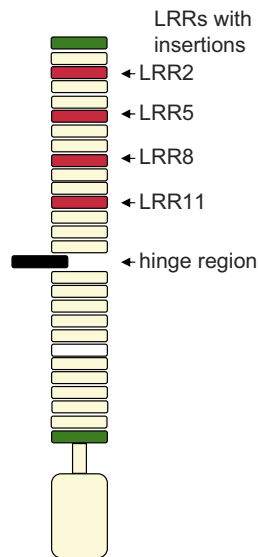


Fig. 4. Structural organization of TLR9. LRRs – leucine-rich repeats; LRRs with insertions are indicated (for details, see text).

we recognized that CpG was prominent in inducing IL-12p40 expression (Heeg and Zimmermann, 2000). Accordingly, co-injection of CpG but not LPS, lipoteichoic acid (LTA) or poly-IC was able to induce a Th1-dominated response in BALB/c mice on challenge from *Leishmania major*. CpG prevented progression of the disease, induction of Th1T cells and a lasting memory. As a consequence, BALB/c mice controlled the infection and did not succumb to progressive disease (Zimmermann et al., 1998). In contrast, LPS (TLR4 ligand), LTA (TLR2 ligand) and poly-IC (TLR3 ligand) failed to induce protection (Albrecht et al., 2004). This CpG effect strongly correlated with induction of IL-12 in vivo. When we analyzed the IL-12p40 induction by various TLR ligands in vitro, we observed that all ligands induced comparable amounts of IL-6 and TNF, yet only CpG was able to induce high IL-12p40 secretion.

In an independent project, we have analyzed the cause for this unique propensity. We found that upon stimulation all ligands activated comparable amounts of secondary signaling molecules (MAP kinases and NFκB translocation), yet only CpG was able to induce significant acetylation of the IL-12p40 promoter, which we showed is a prerequisite for nucleosomal remodeling leading finally to gene activation. In the context of this project, we have analyzed various TLR ligands and CpG ODN sequences for their ability to induce IL-12. We found that profound IL-12 production after stimulation was a central hallmark of CpG DNA but also of imiquimod (TLR7 ligand). All CpG ODN variants which were able to activate cells also showed the ability to induce IL-12p40 nucleosomal remodeling.

Thus, endosomal TLRs seem to differ in their ultimate signal quality from membrane-expressed TLRs. Whether induction of profound acetylation of the IL-12 promoter is due to endosome-associated signaling or due to activation of different signaling cascades like additional IRF activation is not known. Nevertheless, these observations point out that the immunological quality of stimulation with a distinct TLR ligand depends on so far unknown mechanisms (unpublished results; Albrecht et al., 2004; Dalpke and Heeg, 2004). It is obvious that unraveling these mechanisms will help to design specialized new adjuvants with a tailored immune response spectrum.

Conclusions

CpG oligonucleotides represent a new class of adjuvant whose mode of action is widely elucidated. It has been possible to discern uptake of ODN from binding to the receptor (TLR9) and to define the oligonucleotide sequences which determine these effects. Moreover, inhibitory sequences have been found which antagonize CpG DNA. Due to its unique ability to induce Th1-dominated responses, CpG ODN has been qualified as a favorable adjuvant for immunization of cytotoxic T cells like in cancer therapy, or of Th1 cells like in allergy.

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