Toll-like receptor 9: modulation of recognition and cytokine induction by novel synthetic CpG DNAs

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Abstract
Bacterial and synthetic DNA containing unmethylated 2′-deoxyribo(cytidine-phosphate-guanosine) (CpG) dinucleotides in specific sequence contexts activate the vertebrate innate immune system. A molecular pattern recognition receptor, Toll-like receptor 9 (TLR9), recognizes CpG DNA and initiates the signalling cascade, although a direct interaction between CpG DNA and TLR9 has not been demonstrated yet. TLR9 in different species exhibits sequence specificity. Our extensive structure–immunostimulatory activity relationship studies showed that a number of synthetic pyrimidine (Y) and purine (R) nucleotides are recognized by the receptor as substitutes for the natural nucleotides deoxycytidine and deoxyguanosine in a CpG dinucleotide. These studies permitted development of synthetic YpG, CpR and YpR immunostimulatory motifs, and showed divergent nucleotide motif recognition pattern of the receptor. Surprisingly, we found that synthetic immunostimulatory motifs produce different cytokine induction profiles compared with natural CpG motifs. Importantly, we also found that some of these synthetic immunostimulatory motifs show optimal activity in both mouse and human systems without the need to change sequences, suggesting an overriding of the species-dependent specificity of the receptor by the use of synthetic motifs. In the present paper, we review current understanding of structural recognition and functional modulation of TLR9 receptor by second-generation synthetic CpG DNAs and their potential application as wide-spectrum therapeutic agents.

Introduction
The vertebrate innate immune system recognizes and responds to foreign, pathogen-associated molecular patterns (PAMPs) by secretion of various effector molecules. In mammals, at least ten Toll-like receptors (TLRs) are employed to identify PAMPs [1]. Two of them, TLR3 and TLR9, recognize specific nucleic acid motifs present in bacteria and viruses. In response to viral or synthetic double-stranded (ds) RNA, TLR3 stimulates cytokine expression via nuclear factor κB (NF-κB) activation [2]. TLR9 recognizes 2′-deoxyribo(cytidine-phosphate-guanosine) (CpG) dinucleotides, flanked by specific sequences present in bacterial DNA that may be mimicked by synthetic oligodeoxynucleotides [3]. Such CpG motifs trigger signalling pathways that activate various transcription factors, including NF-κB and activator protein-1 (AP-1). This stimulates proliferation of B-cells, the production of the cytokines interleukin (IL)-12, interferon-γ (IFN-γ), IL-6 and tumour necrosis factor-α (TNF-α) and co-stimulatory molecules by monocytes/macrophages, B-cells and dendritic cells [4,5].

TLR9 structure and signalling
Like other TLRs, TLR9 contains an extracellular leucine-rich repeat and a cytoplasmic [Toll/IL-1 receptor (IL-1R)] (TIR) domain [6]. The TIR domain has structural homology with the IL-1R. Unlike other TLRs, TLR9 is mainly expressed in the cytoplasm, and therefore cellular uptake of CpG DNA is required for immune stimulation [7]. The TLRs and IL-1R initiate similar signalling pathways [6]. Generally, ligand-induced dimerization of TLR recruits the adapter protein, MyD88, to the TIR domain. This leads to the engagement of IL-1-associated kinase (‘IRAK’) and another adaptor protein, TNF-receptor-associated factor-6 (‘TRAF-6’) [3,4]. Complex formation activates the stress kinases c-Jun N-terminal kinase (‘JNK’), p38, and inhibitor of κB (‘IκB’), resulting in the activation of the transcription factors AP-1 and NF-κB [4] (Figure 1).

CpG DNA sequence requirement and specificity of TLR9
TLR9 from different vertebrates exhibits specificity for different CpG motifs (Figure 2). Mouse TLR9 prefers a CpG dinucleotide flanked by two purine bases on the 5′-side and two pyrimidine bases on the 3′-side, such as ‘GACGTT’ [8]. Human immune cells optimally recognize ‘GTCGTT’ and ‘TTCGTT’ motifs [9]. A palindromic ‘AACGTT’ motif induces immune responses in both mouse and human systems [10]. Although TLR9 recognizes CpG motifs in various

Key words: CpG DNA, cytokines, immunostimulation, oligonucleotide, phosphorothioate, TLR9. Abbreviations used: AP-1, activator protein-1; CpG, 2′-deoxyribo(cytidine-phosphate-guanosine); ds, double-stranded; IFN-γ, interferon-γ; IL, interleukin; IL-1R, IL-1 receptor; NF-κB, nuclear factor-κB; PAMP, pathogen-associated molecular pattern; PMA, phorbol 12-myristate 13-acetate; PBMCs, peripheral blood mononuclear cells; TIR, Toll/IL-1R; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α; ss, single-stranded.

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**Figure 1** The two members of the TLR family that are known to recognize pathogen-associated nucleic acid patterns

Key signalling components involved are shown. The activated transcription factors up-regulate the expression of a number of cytokines and co-stimulatory molecules. Representative effector molecules and their biological significance are shown. All the cytokines and co-stimulatory molecules shown may be activated through signalling pathways other than the two key pathways shown in the Figure. ERK, extracellular-signal-regulated kinase; IκB, inhibitor IκB; IRAK, IL-1-associated kinase; MAPK, mitogen-activated protein kinase; TRAF6, TNF-receptor-associated factor-6.

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**Influence of CpG DNA phosphate backbone on receptor recognition**

TLR9 recognizes both natural phosphodiester and synthetic phosphorothioate DNAs [8,11]. Immune stimulatory properties of phosphodiester DNA are demonstrated with either bacterial ds DNA or oligonucleotides containing palindromic or poly(dG) sequences that provide stability against nucleases [4,11,12]. As a result of greater metabolic stability, phosphorothioate-modified CpG DNAs have been extensively studied [4,8]. However, patterns of cytokine induction differ distinctly for these various CpG DNAs [4,11]. Phosphorothioate oligonucleotides induce minimal immune responses without the presence of a CpG dinucleotide. The chirality of the phosphorothioate group is generally ignored, although stereoisomers differ in activity [13].

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**Ionic contact sites of CpG DNA backbone with TLR9**

Medicinal chemistry studies illuminate the optimal structure and conformation of CpG DNA for interaction with TLR9 [14,15]. Negative charges are required on the three phosphates on the 5'-side of CpG, as well as on that linking C and G [16]. However, non-ionic internucleotide linkages at the fifth and/or sixth upstream positions significantly enhance activity [17]. Non-ionic linkages in the 3'-flanking sequence have an insignificant effect on activity.

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**Significance of nucleotide conformation for receptor recognition**

The presence of TLR3 and TLR9 receptors in vertebrates suggests that their immune system adapted to recognize both RNA and DNA pathogens through the conformation of nucleic acids. TLR3 specifically recognizes RNA that adopts the C3'-endo conformation [2,3], whereas TLR9 exhibits
Figure 2 | Short immunomers show a strong activation of NF-κB and phosphorylation of p38 in J774 cells, and induce cytokines in both murine and human cell-culture systems

Conventional CpG DNA containing the murine-specific CpG motif shows activation of NF-κB, but not the one containing human-specific CpG motif in J774 cells. Oligonucleotide sequences (detailed hereafter in the legend as 1–5) correspond to: 1, 5′-d(CTATCTGTCGTTCTCTGT)-3′; 2, 5′-d(CTATCTGACGTTCTCTGT)-3′; 3, 5′-d(TGGTG-3′-X-Y-X-3′-TGGC)-5′; 4, 5′-d(TGGTG-3′-X-Y-X-3′-TGGC)-5′; and 5, 5′-d(TGGTG-3′-X-Y-X-3′-TGGC)-5′. (All sequences are phosphorothioate-modified and X and Y represent glyceryl and propyl linkers respectively. See [30] for further details.) (A) Activation of NF-κB in J774 macrophages after stimulation with 10 µg/ml of immunomers for 1 h. Lane 1, media-treated control; lane 2, human-specific CpG DNA (1); lane 3, control non-CpG DNA (5); lane 4, mouse-specific CpG DNA (2); lane 5, lipopolysaccharide at 0.1 µg/ml; lane 6, short immunomer (3); and lane 7, short immunomer (4). (B) p38 phosphorylation in J774 macrophages following activation with immunomers for 30 min at 10 µg/ml concentration. Lane 1, media-treated control; lane 2, mouse-specific CpG DNA (2); lane 3, short immunomer (3); lane 4, short immunomer (4); lane 5, human-specific CpG DNA (1); and lane 6, lipopolysaccharide at 0.1 µg/ml. Total p38 content is shown in lower panel. (C) Concentration-dependent cytokine induction in BALB/c mouse spleen-cell cultures. The symbols denote the following oligonucleotide sequences: □, (2); ○, (1); △, (3); and ▽, (4) for both the upper and lower panels. (D) Induction of IL-12, IFN-γ, IL-6 and IL-10 secretion in human PBMC cultures at 1 µg/ml concentration of short immunomers after 72 h treatment. Immunomer 1, 2, 3 and 4 correspond to oligonucleotide sequences 2, 1, 3 and 4 respectively, as shown above. Reprinted from Biochem. Biophys. Res. Commun., vol. 300, L. Bhagat, F.G. Zhu, D. Yu, J. Tang, H. Wang, E.R. Kandimalla, R. Zhang and S. Agrawal, pp. 853–861, © 2003, with permission from Elsevier Science.

high specificity for d(CpG) dinucleotides with C2-endo conformation. Substitution of either dC or dG with ribonucleosides or 2′-substituted ribonucleosides suppresses activity [16]. The incorporation of 2′-O-substituted ribonucleosides adjacent to the CpG dinucleotide on the 5′-side impairs activity, but has a minimal effect on the 3′-side [18–20]. However, the conformational changes imposed by 2′-O-substituted ribonucleosides distal to the CpG dinucleotide favour recognition and/or interaction with the receptor and increase activity, including cytokine induction [20]. Additionally, the importance of 3′→5′-linkages in CpG DNA is indicated by site-specific incorporation of unnatural 3′-deoxynucleosides in CpG DNA that results in 2′→5′-linkages [20,21].
Role of nucleobases and nucleosides in the flanking sequences on the receptor recognition

Both nucleobases in CpG are required for immune stimulation [22]. In contrast, activity increases on deletion of a nucleobase lying three or more positions to the 5'-side of CpG [22]. A similar deletion in the 3'-flanking sequence does not significantly affect activity, suggesting that nucleobases downstream of the CpG are not involved in recognition [22]. Surprisingly, in certain positions, the entire nucleoside can be dispensed of by substituting with a non-nucleosidic linker [23]. However, the nature and number of linkers substituted influence the resulting immune stimulation, depending on the position of substitution [23].

Though its interaction with CpG DNA is not well understood, TLR9 discriminates between motifs (P1P2CGP3P4) with different preferences in mouse and human. Using an abasic linker (X) at P1–P4 showed that a nucleobase is absolutely required at both P1 and P2 [24]. Surprisingly, X is permitted in place of either P1 or P2, depending on the neighbouring base. It was shown that ‘GXCGTT’ motif has an intermediate activity between those of ‘GACGTT’ and ‘GTTCGTT’ in the mouse cells [24].

Functional groups of cytosine and guanine required for TLR9 recognition

Although flanking sequences play a significant role, CpG is the principal entity recognized by TLR9 in ss or ds DNA. Changes within the dinucleotide that alter its chemical or conformational nature affect recognition by the receptor and subsequent immune responses. A methyl group substitution at the 5-position of cytosine abolishes binding [4,16], and vertebrates use this to distinguish their own DNA from that of infectious bacteria [4]. However, a hydroxy group substituent at this position does not interfere with activity [25].

The 2-keto, 3-imino and 4-NH₂ functional groups of cytosine are involved in recognition [25]. Surprisingly, the latter hydrogen-bond-donating group may be alkylated without the loss of activity [25]. The guanine component of CpG requires its hydrogen-bond acceptor and donor functional groups at the 1-, 2- and 6-positions, but not the nitrogen at the 7-position [25]. The identification of functional groups in CpG required for the recognition and/or interaction with the receptor led to the synthetic alternatives YpG, CpR and YpR, where ‘Y’ and ‘R’ are synthetic pyrimidine and purine analogues respectively [25].

TLR9 reads DNA sequence from the 5'-end

The sequence that is 5' to the CpG dinucleotide is more critical for receptor recognition and/or binding than the sequence on the 3'-side. In fact, an accessible 5'-end of DNA is required for activity, and blocking the 5'-end abrogates activity, suggesting that the receptor reads the CpG DNA sequence from the 5'-end [26–28]. The accessibility of the 5'-end of CpG DNA to the receptor is dependent on the size of the ligand or oligonucleotide conjugated to CpG DNA at the 5'-end. Conjugation of smaller ligands or molecules, such as a phosphorothioate group or mono- or di-nucleotides, at the 5'-end of CpG DNA has an insignificant effect on activity [27]. However, conjugation of a tetramer or longer oligonucleotide via its 5'-end or even a fluorescein molecule at the 5'-end of a CpG DNA significantly interferes with receptor recognition and suppresses activity, although cellular uptake is not affected significantly [27].

Surprisingly, compared with conventional CpG DNAs, 3'→5' linked CpG DNAs (immunomers) induce higher and subtly different cytokine secretion profiles [28].

Enhanced cytokine secretion by immunomers is consistent with their potent activation of NF-κB in mouse macrophage cell lines. In contrast, 5'→5' linked CpG DNAs induce neither NF-κB activation nor cytokine secretion, and TLR9 appears to be unresponsive to DNA containing two appropriate CpG motifs, but lacking an accessible 5'-end [27,28]. However, the cause for different cytokine-secretion profiles with immunomers remains unclear. Multiple accessible 5'-ends may permit rapid binding and dimerization of TLR9 changing the kinetics of activation of NF-κB. With conventional CpG DNAs containing multiple CpG motifs, binding may be limited to one receptor molecule by the single accessible 5'-end.

PO immunomers are the first class of phosphodiester oligonucleotides to induce potent immune responses without the need for polydG or palindromic sequences [29]. Immunomer design not only provides optimal TLR9 recognition characteristics, but also contributes to higher metabolic stability of CpG DNAs and in vivo anti-tumour activity [29].

Overriding sequence specificity of TLR9

Immunomers may contain strands with only 5 or 6 nt each [30]. Importantly, they do not require the ‘PuPu(Py) CGPyPy’ hexameric motif essential for CpG DNA immune stimulation [30]. Surprisingly, the same short immunomers induce high IL-12 and minimal IL-6 secretion in mouse spleen cell (Figure 2) and peripheral blood mononuclear cell (PBMC) cultures [30]. Unlike conventional CpG DNAs, the novel short immunomer design optimally stimulates NF-kB and stress-activated pathways in murine cells, and induces cytokine secretion in both murine and human immune cell cultures (Figure 2).

Conclusions and future perspectives

Significant progress has been made recently in understanding the role of TLR signalling in innate immunity. Therapeutic applications of TLR-signalling agonists and antagonists are being pursued vigorously. Activation of innate responses can provide broad-spectrum protection against numerous pathogens, cancers, allergy and asthma. Although not possessing memory, the innate immune system assists...
the development of strong adaptive immune responses. The ability of CpG DNA to induce predominantly Th1-type responses signifies its application as a universal adjuvant. Undoubtedly, CpG DNA is a powerful tool that can be used to target a wide spectrum of disease indications through modulation of innate immunity.

Recent progress in antisense oligonucleotide chemistry, manufacturing and pharmacology has provided the necessary experience for a rapid advancement of first-generation CpG DNAs to clinical trials. Several of the antisense agents that are currently in clinical trials contain CpG dinucleotides. In general, antisense oligonucleotides are used at severalfold higher concentrations than CpG DNA. To date, several hundred people have been treated for up to 2 years without any evidence of anti-DNA antibody formation or serious adverse safety concerns. While the first-generation CpG DNAs are being evaluated, a number of second-generation immunomodulatory oligonucleotides have been developed for optimum induction of specific cytokine profiles and activation of human and murine immune cells. Medicinal chemistry sheds light on TLR9–CpG DNA interactions in the absence of TLR9 structural information. All the evidence indicates that the receptor recognizes several synthetic motifs besides the natural CpG motif, and that alternative nucleotide-motif-recognition patterns of the receptor produces different cytokine-secretion profiles. Investigations of molecular recognition of modified CpG DNAs by different cells from a wide range of species will illuminate the process further, and permit the design of superior, less toxic immunomodulators for specific applications.

References

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