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Minimal Sequence Requirements for Oligodeoxyribonucleotides Activating Human TLR9

Jelka Pohar,*1 Alenka Kuznik Krajnik,*1 Roman Jerala,*† and Mojca Benčina*†

Synthetic oligodeoxyribonucleotides (ODNs) containing CpG (unmethylated deoxyctydyl-deoxyguanosine dinucleotide) motifs activate endosomal TLR9. The nucleotide sequence, length, and dimerization properties of ODNs modulate their activation of TLR9. We performed a systematic investigation of the sequence motifs of B-class and C-class phosphodiester ODNs to identify the sequence properties that govern TLR9 activation. ODNs shorter than 21 nt and with the adenosine adjacent to the cytidine-guanosine (CG) dinucleotide motif led to a significant loss of the propensity to activate TLR9. The distance between the stimulatory CpG motifs within the ODN fine-tunes the activation of B cells. The minimal ODNs that activate human TLR9 comprise 2 CG dinucleotides separated by 6–10 nt, where the first CpG motif is preceded by the 5′-thymidine and the elongated poly-thymidine tail at the 3′ end of the ODN. The minimal sequence provides insight into the molecular mechanism of TLR9 ligand recognition. On the basis of sequence requirements, we conclude that two binding sites with different affinities for CG are formed in the human TLR9 dimer, with a very stringent binding site interacting with the 5′ CpG motif. The Journal of Immunology, 2015, 194: 3901–3908.

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ingle-stranded DNA with CpG (unmethylated deoxyctydyl-deoxyguanosine) dinucleotide motifs, characteristic of bacteria and virus genomes, activates the immune response against invading pathogens (1). The frequency of the cytidine-guanosine (CG) dinucleotide is strongly underrepresented in the human genome, and ~80% of the cytidine in the CG dinucleotide is methylated, which establishes the recognition of DNA-comprising CpG motifs as the structural pattern for the innate immune response. Synthetic ssDNA fragments, oligodeoxyribonucleotides (ODNs) containing stimulatory CpG motifs, also initiate immune signaling in a TLR9-MyD88-dependent fashion, leading to the activation of transcription factors NF-κB and IRF7 and, consequently, the secretion of cytokines and chemokines by B cells, plasmacytoid dendritic cells (pDCs), NK cells, and macrophages that express TLR9 (2, 3). Membrane receptors—mannose receptor 1 (4), Ig-like receptor KIR3DL2 (5), multilectin receptor DEC-205 (6), and scavenger receptor CXCL16 (7)—enhance internalization and define the route of endocytosis of ODNs.

Several types of CpG ODNs, each with a distinct sequence, secondary structure, and specificity for TLR9 activation (8), are potent activators of Th1-type cytokines and so are used as adjuvants (9, 10). The B-class CpG ODNs (alias K-type) with the CG motif and a phosphorothioate (PTO) backbone are strong stimulators of B cell proliferation as well as the secretion of IL-6, IL-10, and IL-12 through NF-κB signaling (1, 11). A-class ODNs (alias D-type), which are poor stimulators of B cells, contain a palindromic sequence at the center of the ODN and poly-G motif, which can form quadruplexes at each end. These ODNs activate NK cells, and in pDCs, A-class ODNs induce secretion of type I IFNs (IFN-α/β), TNF-α, IL-12, and IFN-γ–IP10 (12–14). C-class ODNs are chimeras of A- and B-class ODNs with one or two CGT motifs at the 5′ end and a CG-rich palindromic sequence at the 3′ end of the ODN. This class of CpG ODNs possesses the immune-stimulatory properties of the A- and B-class ODNs and induces proliferation of B cells and the production of low amounts of IFN-α from pDCs (15, 16). A-, C-, and P-class (17, 18) CpG ODNs form intra- and intermolecular duplexes. These multi-ODN structures are suggested to take a different route of endocytosis and, therefore, induce the synthesis of type I IFNs (17, 19). In addition to unmethylated CpG motifs within the ODN, other factors determine the immune response of TLR9-expressing cells. The position, number, and accessibility of CpG motifs, the nucleotides adjacent to the CG dinucleotide, and the secondary structures of the ODNs influence agonist potency for TLR9 activation (15, 20–23). Moreover, CpG ODNs show sequence-species specificity for the activation of TLR9. GACGTT has been proposed as the optimal TLR9 recognition hexamer for mice (1, 24), whereas GTCGTT is optimal for humans (12) and other vertebrate species (25).

The naturally occurring agonist of TLR9 is ssDNA with a phosphodiester (PD) backbone. To prevent degradation of ODNs by deoxyribonucleases, synthetic ODNs may consist of a partial or complete PTO backbone. Some important differences are noted in terms of TLR9 activation between ODNs based on a PD backbone and those based on a PTO backbone. A base-free PD-deoxyribose homopolymer is a very weak TLR9 agonist (26), whereas the base-free PTO homopolymer does not activate TLR9 but instead competitively inhibits TLR9 activation in the presence of B-class ODN, which is not the case for the PD homopolymer (26). In this study, we aimed to define the sequence and functional features of ODNs that govern TLR9 activation and the minimal sequence requirements for strong ODN stimulation of the immune
response in human B cells and human TLR9-expressing HEK293 cells. We systematically analyzed which sequence motifs in B-class ODNs and C-class ODNs are necessary to effectively activate TLR9. The impact of the nucleotide context and the distance between the CpG motifs, as well as the effect of ODN duplex formation on TLR9 activity, were determined. Finally, we established the minimal ODNs defined by the sequence TCG[6–10]CG[7–19] and a total length of >21 nt. These minimal ODNs induce production of TNF-α in human PBMCs.

Materials and Methods

Cell cultures and reagents

Human PBMCs and B cells were cultivated in RPMI 1640 medium (Life Technologies, Invitrogen) supplemented with 10% (v/v) heat-inactivated FBS (Life Technologies, Invitrogen) at 37˚C in 5% CO₂. Human embryonic kidney cells HEK293, transfected with 10% (v/v) heat-inactivated FBS (Life Technologies, Invitrogen) were cultured in IMDM (Life Technologies, Invitrogen) supplemented with an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene, reporter plasmid expressing firefly luciferase under control of the NF-κB promoter (Invivogen), used 50 ng pUNO-hTLR9-HA plasmid (InvivoGen), 150 ng ELAM1-luc plasmid (Promega) according to the manufacturer's instructions (PolyPlus Transfection). For the TLR9-expressing cells, we used 50 ng pUNO-hTLR9-HA plasmid (InvivoGen), 150 ng ELAM1-luc plasmid (Promega) according to the manufacturer's instructions (Invivogen). Normalized alkaline phosphatase was determined using a QUANTI-Blue reagent according to the manufacturer's instructions (InvivoGen). Normalized relative luciferase activity was calculated relative to the ODN2006 output. Error bars represent the SD obtained from three experimental replicates.

HEK293T cells were transiently transfected with plasmids expressing hTLR9HA (140 ng DNA per well) and an EEA1-mTomato (80 ng). Localization was visualized 48 h post transfection. The hTLR9HA was stained using rabbit anti-HA (H6908; Sigma-Aldrich) and secondary Abs Alexa Fluor 647 (ab’); fragment of goat anti-rabbit IgG (H + L) (A21246, 10 μg/ml; Invitrogen). Successive images excited at 543 nm (fluorescence emission, 560–620 nm) and 633 nm (fluorescence emission, 650–700 nm) were captured using the Leica TCS SP5 inverted laser-scanning microscope on a Leica DMI 6000 CS module equipped with an HCX Plane-Apochromat 63 × oil-immersion objective with NA 1.4 (Leica Microsystems). Images were processed with LAS AF software (Leica Microsystems) and ImageJ software (National Institute of Mental Health, Bethesda, MD).

AlphaScreen TLR9 binding assay

The binding assay was performed in a final reaction volume of 25 μl assay buffer, which contained 50 mM MOPS-NaOH (pH 6.5), 100 mM NaCl, 0.01% (w/v) Tween 20, and 1 μM BSA in a half 96-well plate (PerkinElmer). The ODNs labeled with biotin-TEG at 3’ (Integrated DNA Technologies) were used. In the assay, 5 μl cell lysate (prepared as described below) was incubated with an ODN (≤75 μM final concentration) for 1 h at 23˚C. Anti-HA acceptor beads (20 μg/ml final concentration) were added and incubated for 1 h at 30˚C. Then, streptavidin-coated donor beads (20 μg/ml final concentration) were added and incubated for 30 min at 30˚C before the signals were measured with EnVision (PerkinElmer).

The binding of ODN to wt-tTLR9HA or wt-tTLR9cyclo导弹(eTCPc) after subtraction of intrinsic activity (no ODN) are shown as AlphaScreen units (all n = 3, ± SD; two independent experiments).

HEK293T cells were transiently transfected with plasmid expressing hTLR9HA (735 μg DNA per well) using Lipofectamine Transfection Reagent (Invitrogen). After 24 h, the cells were lysed in 0.2 ml lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 20% glycerol, 1 mM NaF, and 1 mM PMSF) containing a Complete Mini Protease Inhibitor (Roche). Relative luciferase activity was measured using an Orion system reagents (Promega) and the Orion luminometer plate reader (Berthold Detection Systems). Relative luciferase activity was calculated by normalizing each sample’s firefly luciferase activity with the constitutive cOmplete Mini Protease Inhibitor (Roche) for 10 min on ice. Then, cell lysates were centrifuged (10,000 rpm, 10 min, 4˚C).

Internalization of ODNs

The expression of hTLR9HA (735 μg DNA per well) using Lipofectamine Transfection Reagent (Invitrogen). After 48 h, cells were lysed in 0.2 ml lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 20% glycerol, 1 mM NaF, and 1 mM PMSF) containing a Complete Mini Protease Inhibitor (Roche) for 10 min on ice. Then, cell lysates were centrifuged (10,000 rpm, 10 min, 4˚C).

Fluorescence spectroscopy

The dsDNA in solution was analyzed using the Quant-it dsDNA High-Sensitivity Assay Kit (Molecular Probes, Invitrogen). A PicoGreen dye, when bound to dsDNA, fluoresced at 523 nm when excited with 502-nm light. The formation of dsDNA was determined with PicoGreen dye at 37˚C (or at 22˚C) with the Synergy Mx (BioTek) spectrofluorimeter according to the manufacturer’s guidelines.

Cytokine detection

Human PBMCs were extracted from whole blood using density gradient medium Lymphoprep and SepMate tubes (STEMCELL Technologies). PBMCs were cultured in 96-well round-bottom plates (0.2 ml per well; 0.5–1 × 10⁶ cells per well) and stimulated with ODNs. Culture supernatants were collected after 16 h. The secreted human TNF-α and IFN-γ were determined using ELISA (human TNF-α and human IFN-γ Ready-Set-Go ELISA; E Bioscience). Untouched B cells were isolated from PBMCs by negative selection with CD43 beads (Miltenyi Biotec). B cells were cultured in 96-well round-bottom plates (0.5–1 × 10⁶ cells in 0.1 ml per well) and stimulated with ODNs. Cells were treated with human FcR blocking reagent and stained with anti-hCD19–FITC, anti-hCD20–VioBlue and anti-hCD69–PE according to the manufacturer’s instructions (Miltenyi Biotec).

Software and statistics

Program IDT OligoAnalyzer 3.1 (www.idtdna.com/analyzer/applications/ oligoanalyzer/) using the program settings, assuming a temperature of 37˚C, 150 mM Na⁺, and 0.5 mM Mg²⁺, was used to calculate the Gibb's free energy of the ODN homodimer. Graphs were prepared with Origin 8.1.
Results

CpG motif at the 5' end of the ODN is essential for TLR9 activation

The number and position of the CpG motifs of ODN2006PD and ODN2395PD were varied to determine the minimal sequence necessary for activating the immune response of B cells and hTLR9-transfected HEK293 cells. B-class ODN2006PD and, to a lesser degree ODN2395PD (C-class), activated NF-κB/AP-1 of the Ramos-Blue cells (Fig. 1). The well-established B-type agonist ODN2006 consists of three CpG-comprising motifs: a 5' located TCGTCGT motif with two CG dinucleotides, followed by the middle GTCGT sequence, and a GTCGT sequence at the 3' end. We substituted one or more CG sequences with TT, and TLR9 activation (NF-κB/AP-1) of the Ramos-Blue cells was measured after the cells were treated with ODN2006PD and its variants (Fig. 1). A minimum of the 5'-CGTCGT motifs and another CG dinucleotide were required for efficient TLR9 activation (Fig. 1A, ODN-57, -13 compared with -58). The impaired activation of TLR9 was detected with the 16-nt-long ODN (Fig. 1A, ODN-14). A marked decrease in TLR9 activation was observed for the ODN variants featuring substitution of the 5'-CG dinucleotide with TT, GG, or GC, even if the other CG dinucleotides remained intact (Fig. 1B, ODN-3, -19, -20). Similar results for TLR9 activation by the ODN variants (Supplemental Fig. 1A, 1B) were also obtained using hTLR9-expressing HEK293 cells (Supplemental Fig. 1C and 1D demonstrates localization and expression).

We also examined the impact of the CpG motif (5'-TCGTCGT) at the 5' end of the C-type ODN2395PD on hTLR9 activation. Substitution of the first CG with TT or GC led to a strong decrease in TLR9 activation (Fig. 1C, ODN-C2, -C7 compared with ODN2395PD), whereas the second CG dinucleotide was much less important (ODN-C3, -C8 compared with ODN2395PD). However, no TLR9 activation was observed when both 5'-CGs were substituted with TT (ODN-C9).

We examined the effect of the increased number of CG motifs by designing longer ODNs: 32-nt-long ODNs CG1-132-F and CG6-132-F, and 40-nt-long CG6-140-F with 1, 6, and 8 CG dinucleotides, respectively. The activity of CG1-132-F with only a single CG was still 50% of the activity of ODN2006PD. The CG6-132-F and CG8-140-F with six and eight CGs activated TLR9 of the Ramos-Blue cells better than the reference ODN2006PD (Supplemental Fig. 2A). When the guanosines within the GG, GC, and CG dinucleotides, except the 5'-CG, were substituted with C, the efficacy in activating NF-κB signaling decreased significantly (Supplemental Fig. 2A; CG1-132-cont, CG6-132-cont, and CG8-140-cont). The results confirmed the crucial role of the 5'-CG and the requirement of at least another CpG within the ODNPD to activate B cells and hTLR9-expressing HEK293 cells.

The adenosine adjacent to the CG dinucleotide reduces the efficacy of ODNs

For human cells, the species-specific motif that activates TLR9 was proposed to be GTCGTT (12), whereas GACGTT was proposed for mice (1, 24). Therefore, we analyzed the consequences of replacing thymidine with the adenosine adjacent to the CG in the CpG motifs on TLR9 activation. These substitutions decreased TLR9 activation. In particular, the adenosine adjacent to the first CG dinucleotide decreased NF-κB/AP-1 activity by >70% (Fig. 2, ODN-23, -11, -25). Several adenosines are present within the sequence of long ODNs; however, the adenosines that are remote from the CpG motifs in CG6-132-woA and CG8-140-woA had almost no influence on the stimulation (Supplemental Fig. 2B). Nonetheless, substitution of five adenosines within the CG1-132-woA improved TLR9 activation. This finding suggests that the A adjacent to 5'-CG had a strong negative effect on activation of the hTLR9, whereas the adenosines placed elsewhere had only a minor impact.

Separation of the CpG motifs modulates TLR9 activation

Initial analysis of the CpG motifs required for TLR9 activation suggested that the distance between the CpG motifs is not a particularly critical parameter (Fig. 1A, ODN-13, -57). To elaborate the requirements for the distance between the 5', middle, and 3' CpG motif in more detail, we designed ODN2006PD variants with two to nine thymidines between the CpG motifs. As a consequence of the thymidine insertions, the lengths of the ODNs varied from 20 to 34 nt (Fig. 3A). The ODN-5 with only two thymidines between the CpG motifs (20 nt long) preserved 60% TLR9 activity. Insertion of three to eight thymidines between CpG motifs (ODN-7, -8, -6) preserved or even slightly increased the ability of the agonist to activate TLR9 (Fig. 3A). Similarly, an insertion of seven or nine thymidines between the 5' CpG motif and the CG-rich palindrome of ODN2395PD improved the TLR9 activation compared with that of ODN2395PD (Fig. 3B). Initial screening (Fig. 3C) suggested that only two CpG motifs within the ODN are sufficient to activate B cells. Therefore, we analyzed the minimal number of CG dinucleotides and their po...
sition within the ODN that promote TLR9 activation comparable to that of ODN2006PD. Substitution of the second CG within the 5’ CpG motif (5’-TCGTTCGT) of ODN2006 with TT, accompanied by the replacement of the 3’ CpG (GCCTG) with TTGTTCT, was well tolerated, with only a minor decrease in TLR9 activation (Fig. 3D, ODN-53, -54). To summarize, only two CG dinucleotides within the ODN were required for receptor activation. We also determined that the 5’-CG dinucleotide and the CpG motif separated by four to eight thymidines were well tolerated.

**Dimer formation decreases NF-κB/AP-1 activation**

The ssDNA is a ligand for TLR9 (1); however, intra- and intermolecular secondary-structure–forming ODNs stimulate type I IFN secretion (12–16, 19, 27). ODN2395 forms a homodimer mediated by the 3’-CG–rich palindromic sequence. For the ODN2395 dimer, the predicted ΔG was −36.75 kcal/mol. Formation of the dsDNA was monitored using PicoGreen, a dsDNA-specific dye. Variants of ODN2395PD were prepared with modifications in the palindromic segment of ODN2395, rendering the duplex less stable (Fig. 4A, ODN-C4, -C6, -C10, -C5; Supplemental Fig. 2C, TLR9-expressing HEK293 cells). As expected, gradual replacement of the palindromic sequence with thymidines decreased the intensity of fluorescence, which indicates the disrupted stability of the dimer (Fig. 4B, 4D). We observed a negative correlation between the stability of the dimer formation and hTLR9 activation at 37°C and 22°C (Pearson’s correlation coefficient −0.88 and −0.65, respectively) (Fig. 4C, 4D; Supplemental Fig. 2D). This finding reflects the transition from C-type to B-type ODN, which is a more potent activator of B cells (Fig. 4A).

To demonstrate that the decrease in activity is due to the formation of the duplex and the decreased content of single-stranded DNA rather than to a particular sequence, we used a mixture of long ODNs with their reverse complement sequences. Other TLR9 stimulatory ODNs reported in the literature that contain a duplex all formed homodimers. ODNs that form a heterodimeric duplex over the 3’ segment of the ODN with the remaining single-stranded 5’-TCGT motif showed a negative impact of ODN dimerization on TLR9 activation. Although each of the components (CG1-l32-F/R, CG6-l32-F/R, CG9-l32-F/R) stimulated TLR9, the mixture of each strongly impaired TLR9 activation (Supplemental Fig. 2E) (15, 28), with <30% TLR9 activity compared with that of ODN2006PD or the activity of individual monomers (Supplemental Fig. 2F). This result reflects the transition from B- to C-type ODN, demonstrating the preference for single-stranded conformation over the entire length of the ODN.

**The minimal sequence requirements of ODNs for TLR9 activation**

On the basis of these results, we selected pairs of CG dinucleotides and systematically modified their position and distance within the ODN. An ODN comprising only two TCGT motifs had ≥80% of the activity of ODN2006PD (Fig. 5A, ODN-46). Shortening of the poly-T tail at the 3’ end strongly decreased activity (Fig. 5B, ODN-74, ODN-73). Any other nucleotide except T adjacent to the 5’-CG sequence considerably impaired the activation of TLR9 (Fig. 5E, ODN-86 U,-87 deoxyribose backbone, -83, -64, -82). In addition, shifting 5’-CG farther toward the 3’ end, but retaining the length of the ODN, led to a stepwise decrease in receptor activity (Fig. 5C). Therefore, the 5’-CG dinucleotide must be positioned next to the 5’ thymidine. As seen in the ODN2006PD variants (Fig. 1A, ODN-20; Fig. 1C, ODN-C9,-C2,-C7; Supplemental Fig. 2A, long ODNs), as well as for minH, at least two CG dinucleotides were required for TLR9 activation (Fig. 5D). The length of minH and the distance between the CG dinucleotides fine-tune the ODN’s activation potency (Fig. 5F, 5G; Supplemental Fig. 3A, 3B). The CG dinucleotide motifs should be separated by 6–10 nt for the best activity (Fig. 5F). This finding was also confirmed for minH ODNs that are 30 nt long (Fig. 5G). We replaced the poly-T spacer between the CG motifs and the 3’ poly-T tail with poly-C, G, or A sequences. These nucleotide variants restored >80% of the activity of ODN2006PD (Fig. 1A, ODN-C10, ODN-C11, ODN-C12, ODN-C13; Supplemental Fig. 2F).

* FIGURE 2. The thymidine adjacent to the 5’ CpG motif is important for TLR9 activation. Ramos-Blue cells were stimulated with ODN2006PD variants with adenosine next to the CpG motif. ***p < 0.005.

* FIGURE 3. The number and position of CpG motifs within ODN fine-tune TLR9 activation. Ramos-Blue cells were stimulated with (A) ODN2006PD and its variants with two to nine thymidines between the CpG motifs; (B) ODN2395PD and its variants with four to nine thymidines between the CpG motif and the CG palindrome; (C) ODN2006PD variants with 5’-CG and an additional CG dinucleotide; and (D) ODN2006PD variants with four to eight thymidines between 5’-CG and an additional CG dinucleotide. The predicted dimer is underlined. *p < 0.5, ns, p > 0.5.
substitutions considerably diminished the potency of minH to activate TLR9 (Fig. 5H). Cytidines replacing the poly-T spacer or tail had a less detrimental effect than the larger purines A and G. Consequently, the poly-T tail at the 3′ end should be between 9 and 19 nt long. As determined by AlphaScreen assay, the 24-nt-long ODNs with different numbers of CG dinucleotides (ODN2006, minH-75) bound TLR9 with similar affinity regardless of the sequence (Supplemental Fig. 4A). The minH-30 with only the 5′ TCG motif and the minH-64 with the ACG motif at the 5′ end bound TLR9 with lower affinity. The 15-nt-long ODN (minH-70) and poly-T did not bind to TLR9. The internalization of ODNs (ODN2006 and minH-75 or poly-T) to B cells was not sequence dependent, and 5 h after ODN treatment, all Ramos-Blue cells contained both ODNs (Supplemental Fig. 4B, 4C). To summarize, the minimal sequence of minH that activates TLR9 is TCG[T]_{6-10}CG[T]_{10-19} with the length of the ODN ranging from 21 to 30 nt.

Response of human PBMCs and B cells to the minimal ODN

Because internalization and nuclease digestion might play a role in TLR9 activation, we tested in parallel the activation of TLR9 by minH ODN with PD and PTO backbones (Fig. 6A). The responses of the B cells triggered by minH ODN and ODN2006 were comparable. It was found that 5–10 times less ODN with the PTO backbone was needed for the full activation of TLR9 than was needed for ODNs with the PD backbone (Fig. 6A), suggesting that the recognition site is optimized for PTOs.

Substitution of the PTO backbone with the PD backbone for only the 5′-CG dinucleotide in the P-class ODN triggers stronger TLR9 signaling (17). Therefore, we examined whether the type of backbone determines the efficiency of TLR9 activation. The minH ODNs with mixed PD-PTO backbone content were tested on B cells. The minH with the PD-linked 5′-TCGT tetrancleotides, with the rest of the nucleotides linked by PTO (minH-75_{PTO}-CG_{PTO}), was the most efficient activator of B cells (Fig. 6B).

Finally, we analyzed minH ODN efficiency to promote a cytokine response in human PBMCs and B cells (CD43-negative) (Supplemental Fig. 4D, CD19- and CD20-positive cells). MinH^{PTO} was as efficient as ODN2006^{PTO} in stimulating TNF-α, IFN-γ, and IL-6 release from PBMCs. The minH^{PTO} also triggered the secretion of TNF-α at a 4-fold higher concentration compared with the PTO ODNs (Fig. 6C). IFN-α was detected in the presence of the ODN2006 and minH with PD backbone 16 h after the PBMCs were stimulated. ODN2006^{PTO} and minH-75^{PTO} triggered the expression of the cell surface marker CD69 on B cells (Fig. 6D).

In summary, the minimal sequence requirement for ODNs to stimulate human TLR9 is two CpG motifs, separared preferably with the oligo-T spacer and the oligo-T tail at the 3′ end (Fig. 6E). For efficient activation, the CG motifs should be separated by between 4 and 10 nt, and the ODN should be longer than 21 nt. The sequence requirement for the 5′-TCGT motif is very stringent because the substitution of the T next to CG with any other nucleotide strongly reduced the ability to activate TLR9. The requirements for the second CG motif and nucleotides adjacent to the CG dinucleotide are less rigorous, and substitutions of the nucleotides next to CG only mildly affect the efficiency of activating human TLR9. These facts suggest two probable binding sites within the TLR9 dimer with different binding affinities for the CG dinucleotide: one that binds the 5′-TCGT motif and one that should bind less stringently.

Discussion

Synthetic CpG ODNs are potent stimulators of immune cells expressing TLR9; however, different classes of ODNs that differ in terms of their sequence elicit diverse cytokine patterns (1, 11, 15, 16). In this study, we identified the minimal sequence motifs of B-class ODNs that effectively activate B cells, PBMCs, and HEK293 cells transfected with human TLR9. We identified minH by the sequence pattern TCG[ T]_{6-10}CG[ T]_{10-19} that contains two CG sequences separated by a 6- to 10-nt-long thymidine spacer. The first CpG motif starts at the 5′ end of ODN, and at the 3′ end, a poly-T tail is required. The minH ODN should be longer than 21 nt for the efficient activation of TLR9, which might be due to either increased protection against exonucleases or sequence-independent binding to the receptor. The minH^{PTO} ODN activates NF-κB/AP-1 signaling in B cells as efficiently as ODN2006^{PTO}. Although we cannot exclude the possibility that the length requirement and the addition of the 3′ oligo-T tail may be due to stability against the exonuclease, the similar activation of the PTO-based activation by minH and ODN2006 suggests that this is not the case.

The 5′-CG seems to be most important, but not sufficient, for TLR9 activation, as previously determined in the design of the generally used ODN2006^{PTO} and ODN2395^{PTO} (12, 23, 29–31). The backbone modification to 2′-O-methyl ribose within the 5′ CpG motif, but not within the 3′-CG-rich sequence, of the C-class ODN led to the complete loss of IFN-α secretion.
confirming the importance of 5′-CG and deoxyribose in the activation of TLR9. Moreover, CpG ODNs containing a 3′ hairpin structure, but not the 5′, are immunostimulatory (20), which is consistent with the finding that the accessible 5′ CpG motif is necessary for activating TLR9. The binding of ODNs to TLR9 showed weak sequence dependence, and shorter minH ODN and ODN with 5′-ACG motif poorly bound TLR9. Latz et al. (32), Ashema et al. (33), and Kindrachuk et al. (34) identified that ODNs bind mTLR9 in a sequence-independent manner. However, Rutz et al. (35) demonstrated the sequence-dependent binding of ODN to mTLR9. The number of CG dinucleotides within the ODN is another parameter that defines the agonist’s efficacy in activating TLR9 of B cells as well as PBMCs. More than two CpG motifs improve TLR9 activation, which is understood not only for ODN2006 PD and ODN2006 PTO (23, 29) but also for long ODNs. The CD32 and CD40 with six and eight CG sequences activate B cells better than ODN2006 PD (per mole, but not per mass), although three or more CG dinucleotides within a 22- to 25-nt-long ODN does not enhance activity any further (2).

Nucleotides adjacent to the CG dinucleotide are also important for agonist potency and are an integral part of the CpG motif (12), probably reflecting the binding specificity of the site on the TLR9 ECD. Substitution of the thymidine adjacent to the 5′-CG with any other nucleotide strongly reduced the ability to activate human TLR9. In contrast, the T to A substitution next to any other CG had only a moderate negative effect on activation. Moreover, the poly-T tail at the 3′ end of the minH ODN, which defines the length requirement of the ODN, is necessary for efficient activation. The distance between two essential TCGT motifs fine-tunes TLR9 activation. We showed that TCGT sequences four to eight thymidines apart in minH and ODN2006 PD led to the highest TLR9 activity, as shortening or extending the thymidine spacer outside this range decreases the ODN’s efficacy to activate TLR9.

The sequence difference in the A-, B-, C-, and P-classes predominantly defines the secondary structure of ODNs, which, according to the literature, is the main reason for differences in the activation of B cells and pDCs as well as the secretion of different cytokines (36–39). The synthesis of type I IFNs is primarily linked to the A-class ODNs (12–14), most likely owing to the different

![FIGURE 5. The minimal ODN sequence requirements for activation of TLR9. Ramos-Blue cells were stimulated with ODN2006 PD and (A) its variants lacking the last CpG motif; (B) minH ODNs with different T-tail lengths at the 3′ end; (C) minH ODNs with different T-tail lengths at the 5′ end; (D) minH ODNs with only one CG sequence and different T-tail lengths at the 5′ end; (E) minH ODNs with different nucleotides adjacent to the CG motifs; (F) minH ODNs, 24 nt long, with separation of CG sequences from 5 to 12 nt; (G) minH ODNs, 30 nt long, with separation of the CG sequences from 4 to 24 nt; and (H) minH ODNs with a different 3′ tail sequence and a different spacer sequence between the CG motifs. *p < 0.5. ns, p > 0.5; d, deoxyribose.](http://www.jimmunol.org/Downloadedfrom)
cellular internalization pathway. The latter was also associated with the route of endocytosis and the location of ODNs in differently matured endosomes (14, 40). Nanoparticles loaded with B-class CpG ODNs that mimic A- and P-class ODN aggregates are as effective as A-class ODNs at inducing IFN-$\alpha$ in pDCs and PBMCs (36, 41). This finding implies that the propensity of ODNs to internalize into early endosomes, rather than the specific DNA binding motif, drives MyD88/IRF7-dependent signaling and IFN-$\alpha$ synthesis (40). The B-class ODNs, like minH-75, internalized sequence independently; however, the route of internalization is probably not linked to IRF7-dependent activation, which would trigger type I IFNs. The C-class ODN, in addition to the 5'9CpG motif identical to ODN2006, contains a 3'-CG–rich palindrome that participates in forming homodimers (16). The substitutions within the CG-rich palindromic sequence that preserves the palindrome were well tolerated. We demonstrated that it is possible to design an apparently smooth transition from C- to B-type ODNs by modulating the stability of the palindromic segment. Although many ODNs have been investigated, few systematic studies have been conducted to unravel the key sequence determinants that govern activation of TLR9 by ODNs. The substitutions that destroy the palindrome, however, decrease IFN-$\alpha$ secretion and increase the secretion of IL-6 cells in B cells (16). Negative correlation between dimer formation and NF-$\kappa$B/AP-1 activation was also confirmed with long ODNs and C274 ODN (28). Weaker activation by C-type–like ODNs compared with B-type–like ODNs can be explained by binding to only one CpG binding site, leaving the other CpG binding site unoccupied, which leads to less effective activation of TLR9. There are differences in the TLR9 activation between ODNs based on the PD and PTO backbones. Introduction of the nuclease-resistant PTO backbone improved the ODN’s uptake and hindered degradation by nucleases. A disadvantage of PTO modifications is the induction of an immune response independent of the CpG motif (42–44), which obscures the motif of the physiologically relevant agonists. The PTO-based minH ODN chimera comprising PD-based 5'-TCGT maintained the ODN’s species-specific specificity while retaining protection of the ODN against nucleases. Therefore, such chimeric ODNs are as efficient stimulators of immune cells as ODNs with the PTO backbone.

The minimal sequence motif of ODN required for TLR9 activation contributes to elucidation of the recognition specificity of the ligand–TLR9 interactions. Requirements for the minimal ODN motif suggest that at least two nonequivalent sites at the hTLR9 ECD dimer must be present to interact with CG dinucleotides. One CG recognition site of TLR9 must recognize the 5′-CG motif.
and specifically distinguish thymidine as the first nucleotide. The other CpG binding site, where the recognition of CG bases is probably less stringent, is located apart from the first CG recognition site. The best activation of TLR9 is achieved with ODNs with CG dinucleotide motifs separated with a spacer of at least six nucleotides, which corresponds to a distance of ~40–50 Å and is probably the distance between the binding sites on the TLR9 ECD dimer. In addition, the poly-T tail at the 3′ end of ODN that is ≥10 nt long probably serves as an initial nonspecific binding of ssDNA to TLR9 through a negative phosphate backbone and a positive interface on TLR9. Those structural requirements will also support the design of improved activation because we demonstrated that some ODNs, particularly those that are longer, cause stronger activation than ODN2006.

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References


