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Recognition of CpG oligodeoxynucleotides by human Toll-like receptor 9 and subsequent cytokine induction

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Abstract

Toll-like receptor 9 (TLR9) recognizes a synthetic ligand, oligodeoxynucleotide (ODN) containing cytosine-phosphate-guanine (CpG). Induction of TLR9 by CpG ODN activates a signal transduction cascade that plays a pivotal role in first-line immune defense in the human body. The three-dimensional structure of TLR9 has not yet been reported, and the ligand-binding mechanism of TLR9 is still poorly understood; therefore, the mechanism of human TLR9 ligand binding needs to be elucidated. In functional studies of TLR9, phosphorothioate (PTO)-modified CpG ODNs have been utilized because "natural" CpG ODNs consist entirely of a phosphodiester (PD) backbone that is easily degraded by nucleases. However, PTO ODNs do not faithfully recapitulate natural DNA-mediated TLR9 activation.

In this study, we constructed several human TLR9 mutants, including predicted truncated mutants and single mutants in the predicted CpG ODN-binding site. We used these mutants to analyze the role of potential important regions of TLR9 in receptor signaling induced by stable PD-ODNs that we developed. We clarified that both the C- and N-termini of the extracellular domain (ECD) are necessary for the function of TLR9 in human cells, even if only the C-terminal region of mouse TLR9-ECD was activated by CpG ODNs. Next, we identified residues in the C-terminus of TLR9-ECD (H505 in leucine-rich repeat (LRR)-16, H530 in LRR-17, and Y554 in LRR-18) that are essential for hTLR9 activation. Furthermore, we utilized PD-ODN to analyze the function of TLR9 in peripheral blood mononuclear cells and B cells. PD-ODNs showed perfect sequence-dependent TLR9 activation, whereas both CpG and non-CpG PTO-ODN activated TLR9. Hence, our study revealed the specific use of natural PD-ODN to explore the function of TLR9, which is required for its development as a potential therapeutic adjuvant.

Keywords: toll-like receptor 9, oligodeoxynucleotide, immunostimulatory, ligand-binding sites, extracellular domain

Abbreviations: TLR, toll-like receptor; hTLR9, human toll-like receptor 9; mTLR9, mouse toll-like receptor 9; ODN, oligodeoxynucleotide; CpG, cytosine-phosphate-guanine; PTO, phosphorothioate; PD, phosphodiester; ECD, extracellular domain; LRR, leucine rich repeat; PAMPs, pathogen-associated molecular patterns; pDC, plasmacytoid dendritic cells; PBMCs, peripheral blood mononuclear cells; DMEM, Dulbecco's modified Eagle medium; IL-6, interleukin-6; IRS, inhibitory sequence; NF-κB, nuclear factor-kappa B

1. Introduction

The Toll-like receptors (TLRs) are a class of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), as well as play a critical role in the innate immune response to invading pathogens [1–4]. TLR9 is activated by DNA from invasive bacteria and by synthetic oligodeoxynucleotides (ODNs) containing unmethylated cytosine-phosphate-guanosine (CpG) motifs [5]. TLR9 is localized in the early endosome/endolysosome of mainly B cells and plasmacytoid dendritic cells (pDCs) in humans [6]. CpG ODNs stimulate the innate immune system and therefore have potential application in various immune therapies to treat infectious diseases, asthma, allergy, and cancer [7-9].

TLR9 harbors a leucine-rich repeat (LRR) motif at its extracellular domain (ECD) that is necessary for ligand recognition [10], as well as a transmembrane domain for localization [11,12]. The three-dimensional structure of TLR9 has not been reported; therefore, the structural details of the ligand–receptor interaction and any associated conformational changes remain unclear. Several analyses of the functional structure of human TLR9 (hTLR9) have been reported [13–15], but more detailed information information regarding the ligand-protein structure is necessary to pursue the use of TLR9 in adjuvant development.

Studies of TLR9-mediated immune-stimulation have been performed mostly with CpG ODNs consisting entirely or partially of a phosphorothioate (PTO) backbone, because this backbone is more stable and renders higher cellular uptake compared to the CpG ODN-PD backbone [16]. Roberts *et al.* reported that the binding affinity of CpG ODN-PD from different sequences to TLR9 did not correlate with the established species-specific responses to CpG ODN-PTO [17]. In studies examining the structure–activity relationship between different DNA sequences and activation of TLR9 in cells found that CpG ODN-PD is necessary. Li *et al.* reported that ODN-PTO cause TLR9 aggregation, but PD-ODN induces TLR9 dimerization [18]. It is suggested that ODN-PTO do not faithfully recapitulate natural DNA-mediated TLR9 activation [19]. Recently, we reported a nuclease-resistant, natural CpG ODN-PD, CpG ODN2006x3-PD [20]. This ODN-PD contains 9 CpG motifs and is nuclease resistant. These properties contributed to higher signaling activity of CpG ODN-PD stimulation in TLR9-expressing HEK293-xl cells than did CpG ODN-PTO.

In this study, we evaluated a functionally essential region in hTLR9 by using “natural” PD-ODNs, which we developed. Based on sequence prediction, we constructed a truncated form of hTLR9 and analyzed its signaling activity. Unlike the mouse TLR9 (mTLR9) [21,22], truncated hTLR9 was not activated by CpG ODNs. Moreover, to determine whether any of the proposed ligand-binding sites were responsible for TLR9 ligand recognition, we mutated residues that are highly conserved between different species according to homology modeling study [23] and identified an important positive cluster for CpG recognition. Taken together, these data provide structurally important information to clarify the mechanism of TLR9 activation in human cells. In addition, we showed that PD-ODN induces IL-6 secretion via TLR9 activation in both human peripheral blood mononuclear cells (PBMCs) and B cells in a sequence-dependent manner, although both CpG and non-CpG PTO-ODN induce IL-6. By using “natural” PD-ODNs on behalf of PTO-ODNs, we were able to resolve the ligand recognition and conformational change mechanisms of TLR9 to accurately mimic the immune reaction in mammalian cells.

2. Materials and Methods

2.1. Cells and reagents

HEK293-xl-null cells (InvivoGen, San Diego, CA, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 50 units/mL penicillin, 50 mg/mL streptomycin, and 10 μg/mL blasticidin. Frozen PBMCs were purchased from Cellular Technology Limited (Shaker Heights, OH, USA) and thawed according to the manufacturer's instructions. B cells were isolated from PBMCs by positive selection with CD20 cell isolation kits according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). ODNs were synthesized by Fasmac (Kanagawa, Japan), and are enlisted in Supplementary Material Table 1. Anti-HA, anti-Calnexin, and anti-LAMP-1 antibodies were purchased from AbCam (Cambridge, UK); anti IgG-HRP was from Dakocytomation (Glostrup, Denmark); and anti-rabbit IgG-HRP, Alexa-488 anti-mouse, and Alexa-555 anti-rabbit antibodies were from Invitrogen (Carlsbad, CA, USA).

2.2 Plasmid and TLR9 mutant construction

The plasmid containing the TLR9-encoding sequence, pUNO-hTLR9-HA, was purchased from InvivoGen. Site-directed mutagenesis using the QuikChange Kit (Stratagene, La Jolla, CA, USA) was performed. We also constructed truncated mutants as described in Figure 1A by inverse PCR methods.

2.3 Reporter gene experiments

For reporter gene experiments, a firefly luciferase reporter construct with a nuclear factor-kappa B (NF-κB)-encoding gene was generated. HEK293-xl-null cells (3×10^4 cells/well) were transfected in 48-well format in a volume of 300 μL. hTLR9-HA (500 ng) or the indicated mutant plasmids were transfected using LyoVec (InvivoGen) with 500 ng of pNifty-luc (Invivogen), encoding 5 repeats of NF-κB-binding sites with a firefly luciferase reporter gene, and 100 ng of pGL4.74 (Promega, Madison, WI, USA), encoding *Renilla* luciferase. After 24 h, the cells were stimulated with 0.5 μM ODNs, and luciferase activities were determined after an additional 24 h using the Dual Luciferase Reporter Assay (Promega). The data shown are the mean values of triplicates from 1 of at least 2 independent experiments.

2.4 Immunoblotting

HEK293-xl-null (1.0×10^7 cells) were seeded in 10-cm petri dishes and transfected with 3 μg of the indicated hTLR9-HA vector. After 48 h, cells were collected and rinsed twice with PBS and then lysed with RIPA buffer (Thermo Scientific, Rockford, IL, USA) on ice for 30 min. Lysates were cleared by centrifugation at 12,000 × *g* for 5 min. Equal amounts of lysates were fractionated by 4–12% SDS-PAGE (NuPAGE, Invitrogen) and then electrotransferred to PVDF membranes (Invitrogen). The membranes were blocked with PBS containing 0.5% (w/v) skim milk (Millipore) and 1% (v/v) Tween-20. Cross-reactive bands were visualized using chemiluminescence (Millipore) on X-ray film.

2.5 *Immunoprecipitation and pull-down assay*

Cell lysates were purified using anti-HA antibodies immobilized on Protein A Mag Sepharose (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions. Eluates from the immunoprecipitation were used for pull-down analysis after pH adjustment to an endolysosomal environment using 1 M Tris-HCl (pH 5.0) buffer. A final concentration of 10 μM biotinylated CpG ODN was added to 20 μL of eluates, and the mixture was then incubated for 2 h at 4°C. Subsequently, 20 μL of streptavidin-agarose Dynabeads (Invitrogen) was added, and the mixture was incubated for an additional 2 h at 4°C. Beads were washed 3 times with RIPA buffer and eluted with lysis buffer for immunoblot analysis.

2.6 ELISA

Human PBMCs and $CD20^+$ B cells were seeded in 96-well plates at 5×10^6 and 5×10^5 cells, respectively. CpG ODNs were added at a final concentration of 0.5 μM to the cell culture medium. The cells were then incubated at 37°C for 48 h, and the supernatants were collected and stored at -20°C until further analysis. The IL-6 secretion level was measured with the Human interleukin-6 (IL-6) Ready-Set-Go Kit (eBioscience, San Diego, CA, USA) according to manufacturer's instructions.

3. Results

3.1 Loss of signaling activity in the truncated hTLR9

The proteolytic cleavage of TLR9 is prerequisite for signal transduction in mouse [21,22]. However, it is still unclear whether proteolytic cleavage of TLR9 occurs in human. Thus, we constructed the predicted truncation mutants of hTLR9 (Fig. 1A) and expressed them in HEK293-xl-null cells. Mutant A contains the C-terminal region and mutant B contains both the predicted cleavage site (undefined region, UDR) and the C-terminal region. The signaling activity of mutants A and B induced by the stimulation of CpG ODN-PTO was reduced by approximately 65% compared to hTLR9-WT (wild type). Interestingly, we detected a similar level of signaling activity from mutant C, which consists of only the N-terminal region of hTLR9. Furthermore, stimulation with unstimulatory GpC ODN-PTO totally abolished the signaling activity of the mutants (Fig. 1B). No significant difference in the expression level and localization was observed between full-length hTLR9 and the truncation mutants (Supplementary Material Fig. S1), suggesting that the loss of signaling activity in these mutants was not caused by a lower expression level or improper localization of the mutant proteins. Moreover, the pull-down assay using biotinylated CpG ODN-PTO revealed that all mutants could bind CpG ODN-PTO (Fig. 1D) and GpC ODN-PTO (data not shown). This implies that the loss of signaling activity in these mutants is not due to the loss of binding to CpG ODN-PTO.

We also stimulated these mutants with a nuclease-resistant CpG ODN-PD that we developed recently [20] and demonstrated that all of the truncation mutants lost their signaling activity with nuclease-resistant CpG ODN-PD stimulation (Fig 1C). Both the N- and C-termini of hTLR9 ECD are able to bind to CpG ODN-PD (Fig 1D). This implies that the loss of signaling activity in these mutants is not due to loss of binding to ODN. We confirmed this finding by NF-κB detection of deleted LRR 2, 5, and 8 mutants that occupied N-terminus of hTLR9 ectodomain. LRR deletion mutants totally disrupt TLR9 signaling in response to CpG ODN stimulation

(Supplementary Fig S2.). These results suggest that both the C- and N-termini of the hTLR9-ECD are required for signaling activity.

3.2 H505, H510, and Y554 are functionally essential residues for TLR9 activation

We further tried to identify the sites on TLR9 that are essential for signaling activity. Ten amino acid residues in the C-terminal region of the ECD that are involved in the binding of CpG ODN have been predicted based on homology modeling [23]. These amino acid residues are positively charged and are highly conserved among species. We performed alanine scans of these residues and examined the effects of the mutations on receptor signaling activity. Addition of CpG ODN-PTO and CpG ODN-PD disrupted the signaling activity in cells expressing 8 site-directed TLR9 mutants (R481A, N483A, H505A, Q510A, H530A, K532A, Y554A, and Q557A) (Fig. 2A, B). Mutation of H505, H510, and Y554 almost totally abrogated activity when CpG ODN-PD was used as the TLR9 ligand (Fig. 2B). The signaling activity of these mutants was sequence dependent, where the unstimulatory GpC ODN did not possess a significant NF-κB activity. Furthermore, the loss of signaling activity in these mutants was not due to a difference in the expression level or ODN binding (Supplementary Fig. S3. A, B). Therefore, these amino acid residues are essential for signaling activity and likely form ligand recognition sites in TLR9. This result supplies the information regarding the ligand recognition site in TLR9 that mediates receptor activation, as depicted in Supplementary Fig. S3.C.

3.3 ODN-PD induce IL-6 secretion in human primary cells in a sequence-dependent manner

In HEK293-xl-TLR9 cells, our findings suggested that there is no difference in the mechanism by which hTLR9 recognizes CpG ODN-PTO and CpG ODN-PD. However, CpG ODN PD showed a clear difference between active TLR9 and inactive TLR9 mutants. To better understand this finding, we examined the effect of the backbone on hTLR9 expressing human primary cells, PBMCs and B cells. We stimulated human PBMCs and $CD20^+$ B cells, which are isolated from PBMCs, with CpG ODN-PTO and CpG ODN-PD and examined the level of IL-6 production. The level of IL-6 induced by CpG ODN-PTO was much higher than of the level induced by CpG ODN-PD in PBMCs and B cells (Fig.3A). We further clarified whether this difference was sequence-dependent. Surprisingly, IL-6 secretion was induced by only GpC ODN-PTO, but not GpC ODN-PD in PBMCs and B cells (Fig. 3B). The IL-6 level induced by GpC ODN-PTO was almost half of that induced by CpG ODN-PTO in PBMCs. To confirm whether IL-6 secretion induced by GpC PTO is mediated by TLR9, we utilized TLR9 inhibitory sequence 869 (IRS 869) simultaneously with both CpG and GpC ODN [24,25]. We confirmed that IRS 869 inhibits TLR9 signaling with both CpG ODN-PTO and ODN-PD (Fig. 3C). This inhibition did not appear when we introduced IRS 661, which is a TLR7 inhibitor [24]. As we predicted, IRS 869 also inhibited IL-6 secretion from PBMCs stimulated only by GpC ODN-PTO (Fig. 3D). These data imply that the PTO backbone itself induces IL-6 secretion in human PBMCs and B cells, and that ODN-PD mediates IL-6 in a sequence-dependent manner though TLR9 in human primary cells, while ODN-PTO mediates IL-6 in a sequence- and backbone-dependent manner.

4. Discussion

Human TLR9 is highly similar to mouse TLR9 (75% homology), but human TLR9 recognizes a different CpG sequence from the sequence recognized by mouse TLR9. The optimal mouse CpG motif is an unmethylated CG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines. Human peripheral blood mononuclear cells (PBMCs) are strongly activated by CpG ODNs containing GTCGTT as the core motif [26]. Proteolytic cleavage has been reported to transform mouse TLR9 into a functional form [21,22,27]. Truncated C-terminus of mouse TLR9 is essential to generate functional receptor signaling. However, to our knowledge, this truncation is less frequently reported in human TLR9. Moreover, homology around the cleavage site (E440 in mouse TLR9) is less than 30% between human and mouse. In this study, we generated truncated forms of hTLR9 that are similar to truncated forms of mTLR9, and we did not observe optimum function of these truncated mutants after stimulation with CpG ODN-PTO. Furthermore, truncated mutants of human TLR9 totally lost their responsiveness to CpG ODN-PD. These data suggest the necessity of both the N-and C-termini of hTLR9-ECD in generating the signaling response. Our observation is in accordance with that of a previous study [18] and leads us to the questions of whether this truncation is essential for hTLR9, if it might be located in a different site from that of mTLR9, and whether the truncation has less effect on the signaling response of TLR9.

Our findings also indicate that the ligand-binding mechanism of TLR9 is similar to that of TLR3. Crystal structure of TLR3 shows that double-strand (ds) RNA binds to TLR3 in both the C-and N-termini of TLR3-ECD by multiple binding sites in each terminus [28–30]. Recently, Qi *et al*. reported regulation of TLR3 stability and endosomal localization regulated by cleavage of TLR3, but signaling activity is not regulated by cleavage [31]. Therefore, our finding supported collective information on the functional receptor ligand-binding sites activation mapping.

The functional structure of TLR9 is not fully understood because its three-dimensional structure is not yet known. Several researchers have claimed that there are essential amino acid residues in the N-terminus of hTLR9 [13–15]. In contrast, only the D535/ Y537 double mutant is reported to be involved in TLR9 signaling in the C-terminus of hTLR9 [32]. Our study showed the pivotal role of 8 amino acids in the C-terminus of hTLR9. Of those 10 residues predicted previously by homology modeling [23], 8 residues were found to be involved in TLR9 binding, leading to receptor signaling. These residues were distributed equally, with 2 residues each in LRR 15,16, 17, and 18 of TLR9 by homology prediction. Replacing H505, Q510, H530, and Y554 with alanine was sufficient to negate TLR9 stimulation, indicating that an interaction between these amino acids with CpG ODN is essential for TLR9 activation. Based on the results of our mutational analysis, we drew the mutated residue in the TLR9 homology model (Fig. 2C). H505, H530, and Y554 were vicinally oriented and formed positively charged clusters with which negatively charged ODN could interact. This finding suggests that a negative charge from the backbone phosphate group or sulfate group on CpG ODN occupies a position in close proximity to residues 505, 530, and 554 in the ligand-receptor complex. A histidine imidazole ring can be promoted under mildly acidic condition, suggesting that TLR9 would signal in acidic pH as endolysosome/lysosome condition.

CpG ODN-PTO is utilized for its nuclease-resistant activity that enhances the ODN uptake inside the cell and to avoid the cytokine secretion in response to natural CpG ODN-PD. In spite of that, CpG ODN-PTO is also assumed to induce a non-specific reaction that causes antibody production and safety concerns [33] (Supplementary Material Fig.S3). In this study, we detected different a activation signal from our CpG ODN-PD and CpG ODN-PTO, both in HEK293-xl-null and PBMCs. In HEK293-xl-null cells, CpG ODN-PTO induces high levels of NF-□B activity on TLR9 mutants compared to CpG ODN-PD. Li *et al.* reported that the ODN backbone induced a different TLR9 conformational state [18]. PD-ODN ligand contributes to the dimerization state of TLR9, whereas PS-ODN binding results in the formation of large aggregates of TLR9 [18]. Additionally, this different signaling capacity is also regulated by the hypersensitivity of TLR9 binding to the PTO backbone instead of to the PD backbone [25]. Hence, we observed higher activity induced by CpG ODN-PTO compared to that induced by CpG ODN-PD in any mutants, including N-terminal hTLR9. However, this higher activation of TLR9 by CpG ODN-PTO stimulation might lead to biased interpretation to specify which mutants are highly essential for TLR9 signaling activity compared to CpG ODN-PD. In PBMCs, the difference is not only affected by TLR9 oligomeric state likelihood, but also by the reactivity of the PTO backbone usage that allow IL-6 secretion. Another group also showed that the backbone allowed IL-6 secretion by PBMCs independently of CpG motif, whereas our CpG ODN-PD strictly limited its responsiveness to CpG presence [16,34]. Several other reports suggest that CpG ODN-PTO can activate B cells in a CpG-sequence-independent manner [35–37]. These findings suggest that PTO itself binds non-specifically to various proteins in cells, including transcriptional regulators, and hence induced IL-6 production via TLR9 and in a TLR9-independent manner.

In conclusion, we defined a functionally essential region in hTLR9. For full activation, truncation of both the N- and C-termini is necessary. H505, H530, and Y554 formed the receptor-ligand recognition site. CpG ODN-PD also displays specific recognition in TLR9 that avoid false-positive interactions with CpG ODN-PTO; therefore, our ODN-PD could be a versatile ligand for analyzing the ligand recognition mechanism of TLR9.

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FIGURE LEGENDS

Fig 1. Activity and CpG binding of truncated TLR9

(A) Truncation scheme of hTLR9 mutants. NF-κB activity of truncation mutants with CpG ODN-PTO (B) and CpG ODN-PD (C) stimulation. The asterisk (*) denotes $p < 0.05$. The relative NF-κB activity is the luciferase activity of the ligand-stimulated receptor divided by the luciferase activity of the unstimulated receptor. (D) CpG ODN-PTO and CpG ODN-PD pull-down assay of truncation mutants using HEK293-xl-null cells transfected with HA-tagged hTLR9, lysed, and immunoprecipitated with anti-HA antibodies.

Fig 2. Critical sites of signaling activity in the LRR 15 to 18 in hTLR9 ECD

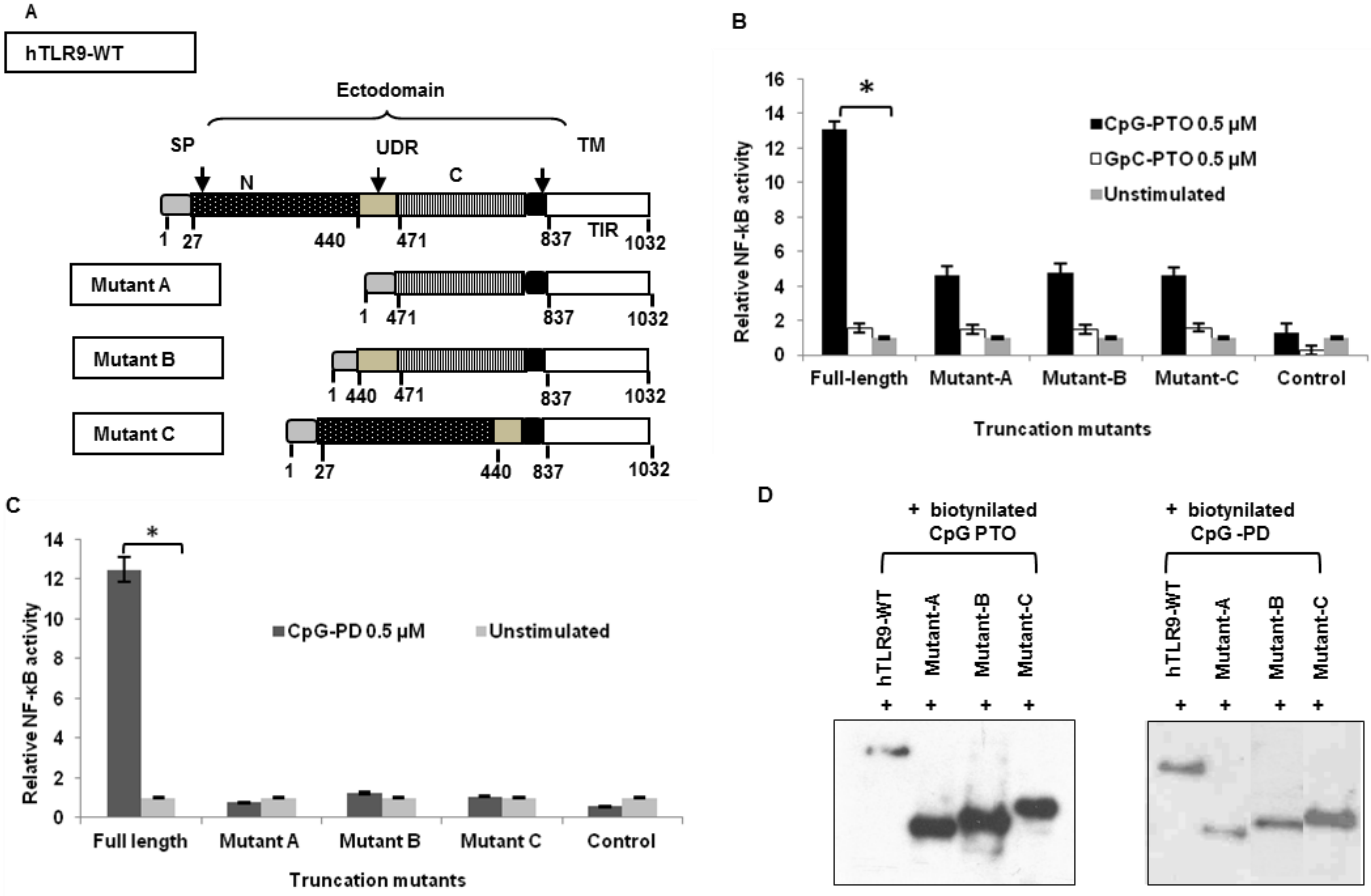
NF-κB activity of mutants of predicted critical sites, stimulated with CpG ODN-PTO (A) and CpG ODN-PD (B). (C) Predicted structure of residues that are essential for receptor signaling activation are marked with red, residues that are less affected by mutation are marked with yellow, and residues that are not affected by mutation are marked with green. White circle indicates the possible cluster for receptor ligand recognition. Predicted structure developed based on homology modeling [9]

Fig 3. IL-6 secretion by PBMCs and B cells with different sequences and backbones

Cells were incubated with the following CpG ODNs: (A) GpC ODNs, (B) CpG ODNs +IRS ODNs, and (C) GpC ODNs + IRS ODNs (D). The supernatants were harvested and IL-6 was measured by ELISA. The graphs represent mean and standard error. The asterisk (*) denotes $p < 0.05$. Experiments were performed in triplicate using 3 individual experiments (for human PBMCs) and in triplicate using 2 individual experiments (for anti-$CD20^+$ B cells).

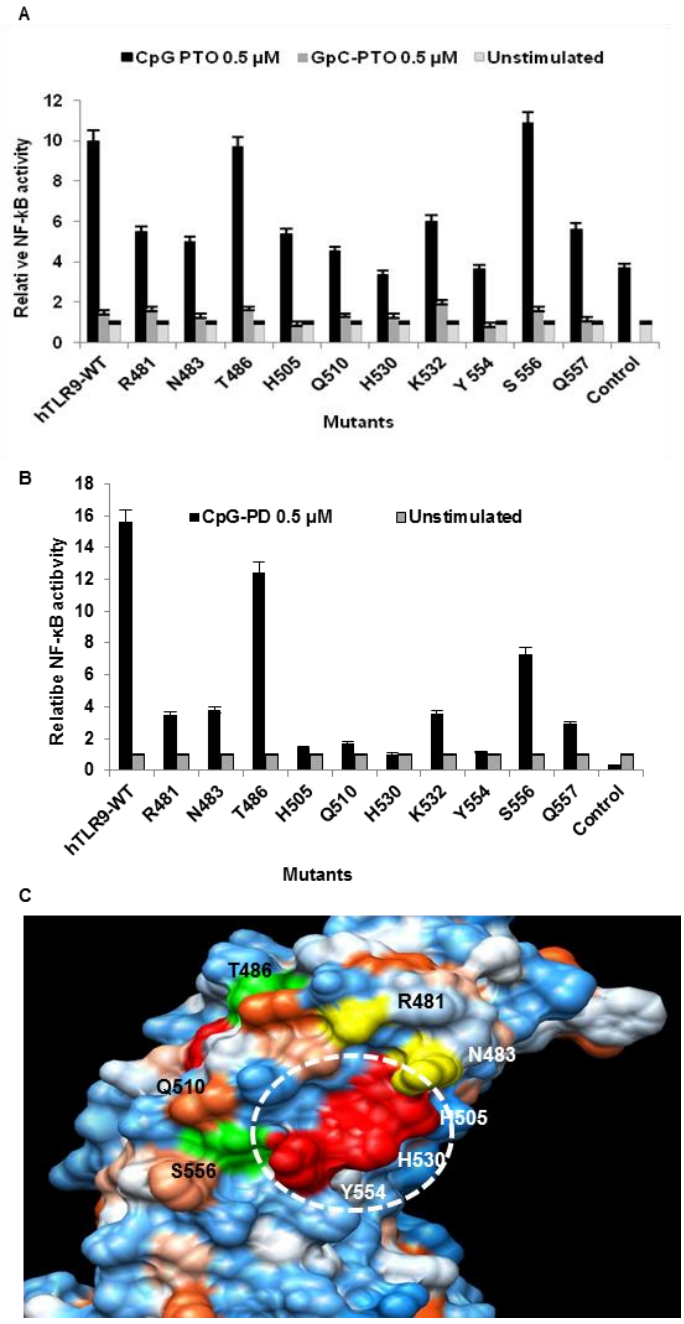
Figures/Tables

Fig. 1



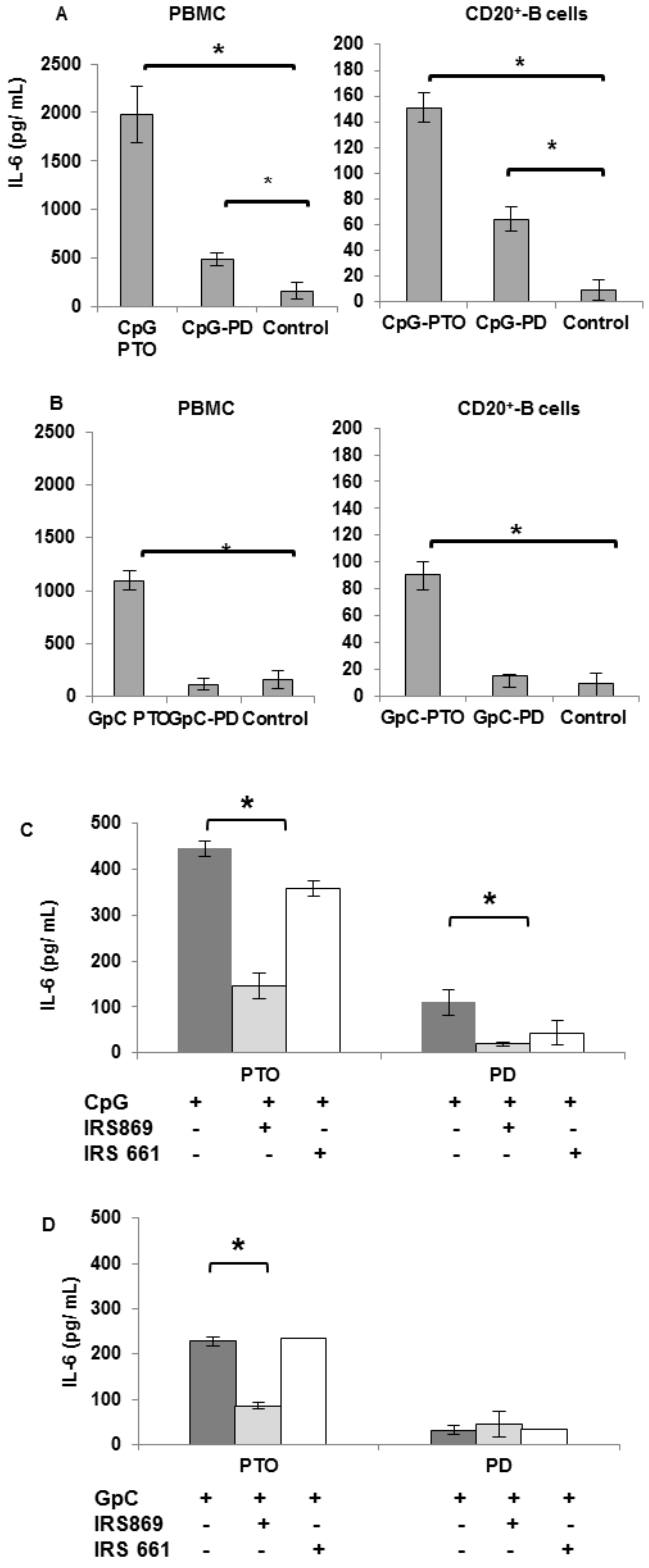
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Fig. 2



(Single column 90 mm)

Fig. 3



(Single column 90 mm)

Highlights

- Both C- and N-termini of human toll-like receptor 9 (hTLR9) extracellular domain are necessary for receptor signaling activation.
- His505, His530, and Tyr554 formed receptor-ligand recognition sites of hTLR9 extracellular domain.
- “Natural” phosphodiester-based oligonucleotide containing cytosine-phosphate-guanine (CpG ODN-PD) acts in a sequence-dependent manner to stimulate hTLR9 in human primary cells, while phosphorothioate-modified CpG ODN activates hTLR9 in a sequence-independent manner.

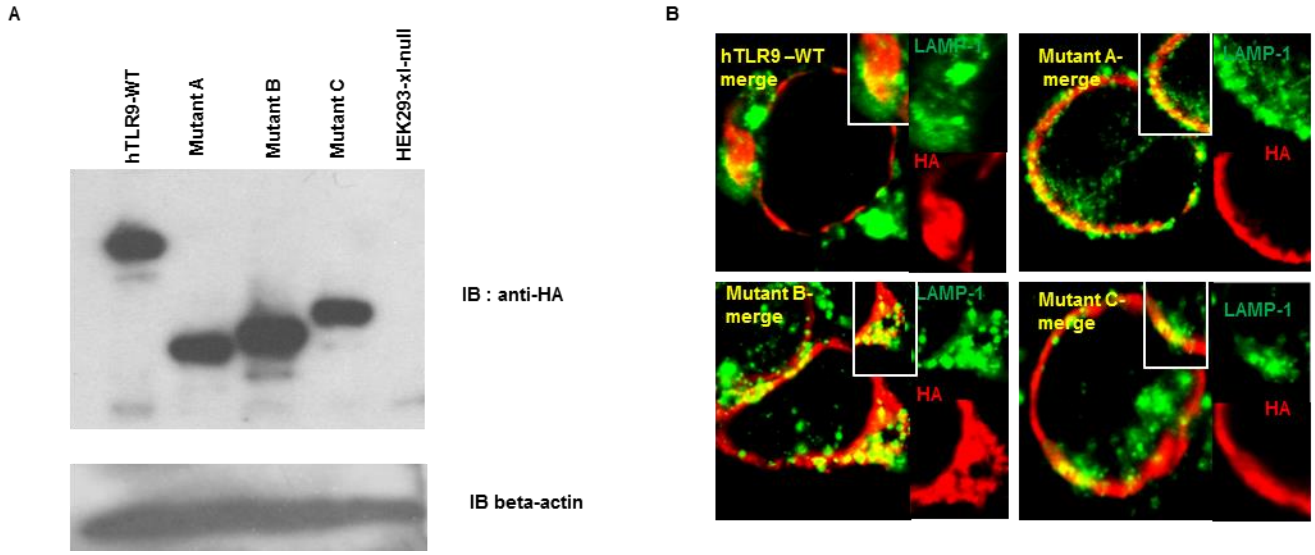
Supplementary Material

Supplementary Material Table 1

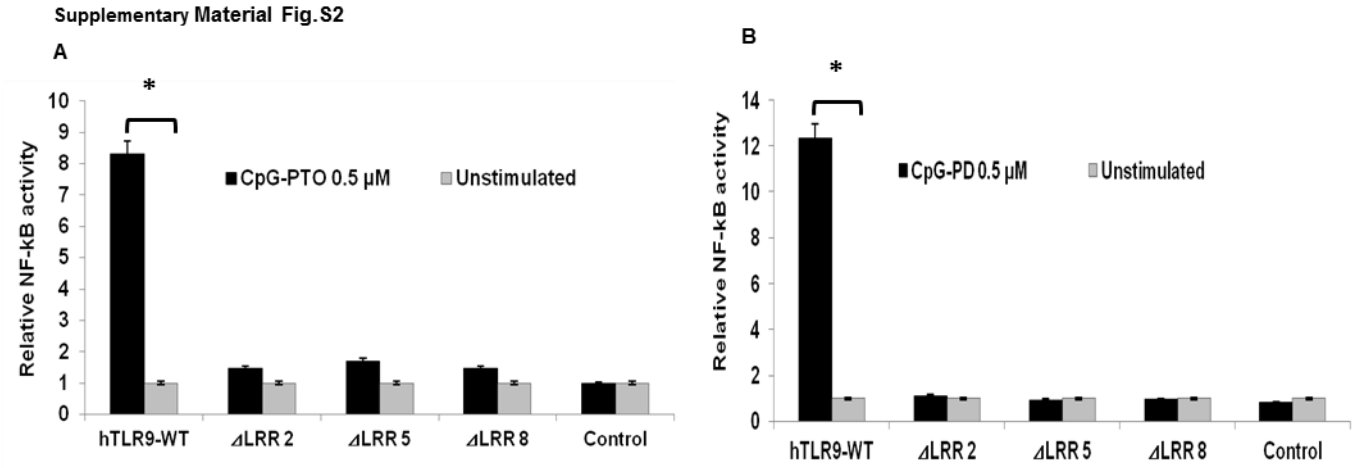
No	ODN	Sequence	Backbone
1	CpG-PTO 2006	5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'	Phosphorothioate
2	GpC-PTO 2006	5'-TGCTGCTTTTGTGCTTTTGTGCTT-3'	Phosphorothioate
3	CpG-PD 2006	5'-tcgtcgttttgtcgttttgtcgtt- tcgtcgttttgtcgttttgtcgtt- tcgtcgttttgtcgttttgtcgtt-3'	Phosphodiester
4	GpC- PD 2006	5'-tgctgcttttgtgcttttgtgctt- tgctgcttttgtgcttttgtgctt- tgctgcttttgtgcttttgtgctt-3'	Phosphodiester
5	IRS 661	5'-TCCTGGAGGGGTTGT-3'	Phosphorothioate
6	IRS 869	5'-TGCTTGCAAGCTTGCAAGCA-3'	Phosphorothioate
7	Random	5'-GTCAGACAGTCTGTGTTGTC-3'	Phosphorothioate

Table 1. List of ODN used in this experiment

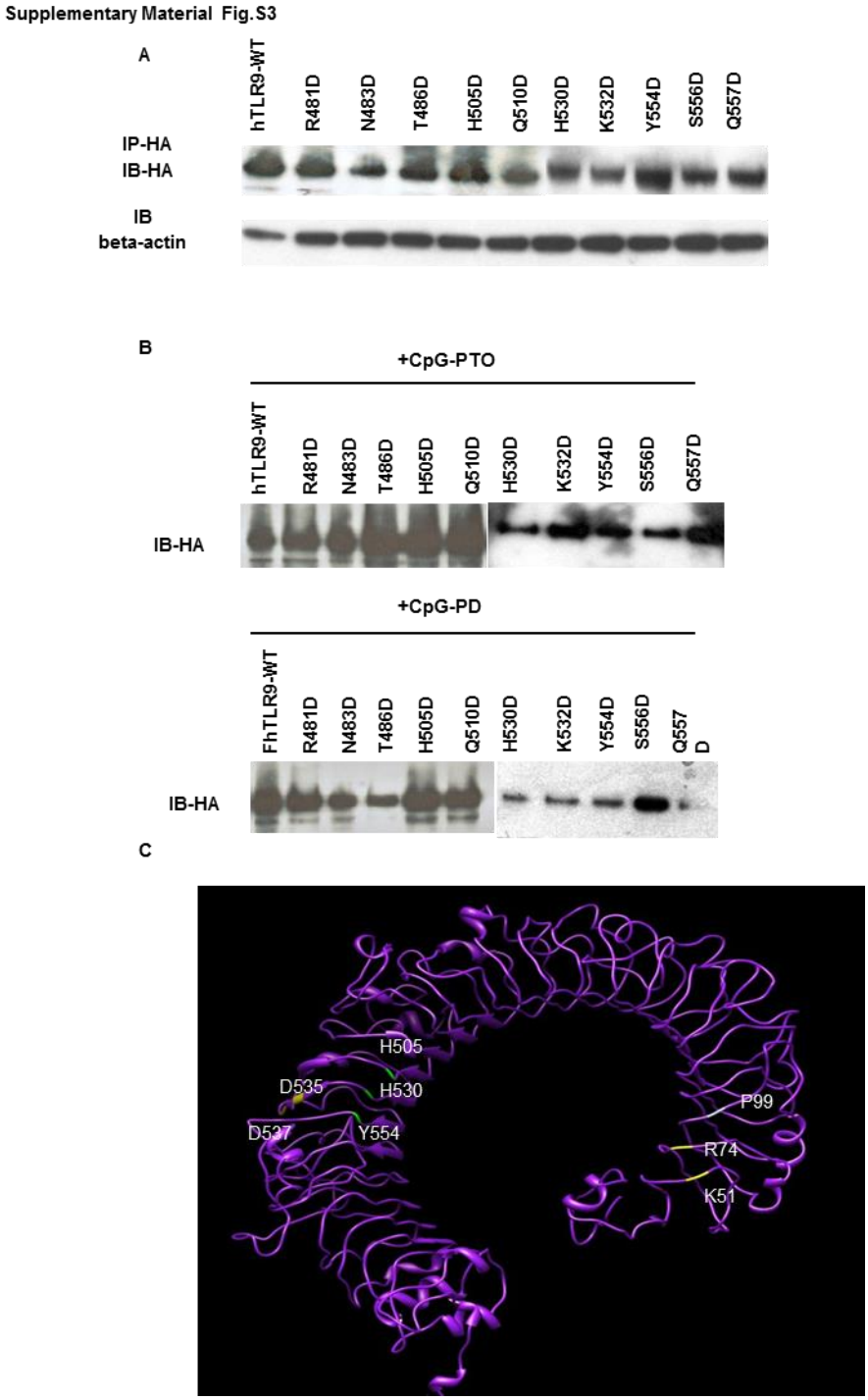
Supplementary Material Fig.S1



Supplementary Material Fig. S1. Truncation of hTLR9 analysis (A) All truncation mutants of the protein express at a level similar to that of the full-length protein. (*Top*) HEK293-xl-null cells were transfected with full-length, HA-tagged hTLR9 and hTLR9 deletions and were then lysed, immunoprecipitated, and immunoblotted with anti-HA antibodies. (*Bottom*) Cell lysate immunoblotted with anti-β-actin antibodies as the loading control. (B) Localization of the mutants in the presence of CpG ODN-PTO. HEK293-xl-null cells were transfected with hTLR9, and the respective mutants were stimulated with CpG ODN-PTO and stained with anti-HA and anti-LAMP-1 for lysosome visualization. The experiments were performed in duplicate.



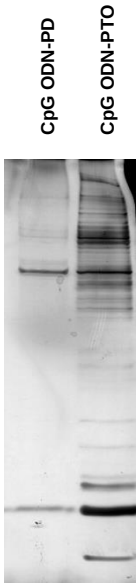
Supplementary Material Fig. S2. N-terminal LRR deletion abolished TLR9 receptor signaling. NF-κB activity of mutants of LRR 2,5, and 8 deletion in N-terminus of hTLR9 ectodomain, stimulated with CpG ODN-PTO (A) and CpG ODN-PD (B).



Supplementary Material Fig. S3. Ligand-binding sites essential for hTLR9 signaling (A) All mutants in the C-terminal region of the hTLR9 ECD express at a level similar to that of the full-length receptor. (*Top*) HEK293-xl-null cells were transfected with full-length HA-tagged hTLR9, and the mutants and the transfected cells were lysed, immunoprecipitated, and immunoblotted with anti-HA. (*Bottom*) Cell lysate immunoblotted with anti-β-actin antibodies as the loading control. (B) Pull-down assay of all C-terminal region mutants of the hTLR9 ECD by using HEK293-xl-null cells transfected with HA-tagged hTLR9. (C) Predicted structure of

TLR9 modeling. Colors denote essential residues for receptor signaling examined through site-directed mutagenesis in human by this study (green), published (light blue), and in mouse (also published) (yellow). Structure developed using I-TASSER program.

Supplementary Material Fig.S4



Supplementary Material Fig. S4. CpG ODN-PTO interacts with many DNA-binding protein compared to CpG ODN-PD. Silver stain analysis of HEK293-xl transfected with hTLR9 lysate pulled down using biotinylated CpG ODN also Streptavidin magnetic beads.