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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). Activation of TLR9 by CpG ODN induces 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 (hTLR9) ligand binding needs to be elucidated. In this study, we constructed several hTLR9 mutants, including 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 hTLR9 in receptor signaling induced by phosphorothioate (PTO)-modified CpG ODN and CpG ODNs only consist entirely of a phosphodiester (PD) backbone, CpG ODN2006x3-PD that we developed. We found truncated mutants of hTLR9 lost the signaling activity, indicating that both the C- and N-termini of the extracellular domain (ECD) are necessary for the function of hTLR9. We identified residues, His505, Gln510, His530, and Tyr554, in the C-terminal of hTLR9-ECD that are essential for hTLR9 activation. These residues might form positive charged clusters with which negatively charged CpG ODN could interact. Furthermore, we observed ODN–PD induced interleukin-6 (IL-6) through TLR9 in a CpG-sequence-dependent manner in human peripheral blood mononuclear cells and B cells, whereas ODN–PTO induced IL-6 in a CpG-sequence-independent manner. These finding are relevant for the mechanism of hTLR9 activation by CpG ODNs.

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]. In human, 10 kinds of TLRs, TLR1-10, have already been identified and classified into subfamily based on PAMPs type. TLR1, 2, and 6 are grouped into lipoprotein or lipopeptides recognizing-PAMPs, TLR4 and 5 are classified into flagellin and lipopolysacharide-PAMPs subfamily, while TLR3, 7, 8, and 9 are crucial for nucleic acid PAMPs recognition. 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 regarding the ligand–protein structure is necessary to pursue the use of hTLR9 in adjuvant development.

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

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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]. However, the ODN–PTO is associated with problems such as non-specific binding that may lead to bias when exploring the hTLR9 recognition mechanism. Roberts et al. reported that the binding affinity of CpG ODN–PD to TLR9 did not correlate with the established species-specific responses to CpG ODN–PTO [17]. Li et al. reported that CpG ODN–PTO cause TLR9 aggregation, but CpG ODN–PD induces TLR9 dimerization [18]. These results suggest that CpG ODN–PTO is in effect to TLR9 activation with the different mechanism from CpG ODN–PD. Wagner hypothesized that CpG ODN–PTO lead to the TLR9 clustering that might generate TLR9 hyperactivation hence CpG ODN–PTO do not faithfully recapitulate natural DNA-mediated TLR9 activation [19]. Therefore, study of TLR9-ligand recognition using ODN–PD gain importance. However, ODN–PD is rapidly degraded by nuclease and much less is known about ODN–PD-induced TLR9 activation. Recently, we reported a stable 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 CpG ODN–PTO and CpG ODN–PD, which we developed. First, based on sequence prediction, we constructed a truncated form of hTLR9 and analyzed its signaling activity to investigate that whether truncated mutants of hTLR9 activate by CpG ODNs. Truncated mutants of hTLR9 lost the signaling activity. Second, 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 [21] and identified an important positive cluster for CpG recognition. Taken together, these data provide structurally important information to clarify the mechanism of hTLR9 activation. In addition, we found that CpG ODN–PD induces interleukin-6 (IL-6) secretion via TLR9 activation in both human peripheral blood mononuclear cells (PBMCs) and B-cells in a CpG-sequence-dependent manner, although PTO–ODN induce IL-6 in a sequence-independent manner. By using CpG ODN–PD on behalf of CpG ODN–PTO, we were able to resolve the ligand recognition of hTLR9 and hTLR9-mediated pro-inflammatory cytokine induction mechanism 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 (here referred as hTLR9-WT) 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 Fig. 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-WT (500 ng) or the indicated mutant plasmids were transfected using LyoVec (InvivoGen) with 500 ng of pNifty-luc (Invivogen), encoding five 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 or unstimulated, and luciferase activities were determined after an additional 24 h using the Dual Luciferase Reporter Assay (Promega). The stimulation activity was expressed as the relative NF-κB activity by ODNs relative to that unstimulated. The data shown are the mean values of triplicates from one of at least two independent experiments. Control is un-transfected with TLR9 encoding vector in HEK293-xl-null cells.

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 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 three 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 IL-6 Ready-Set-

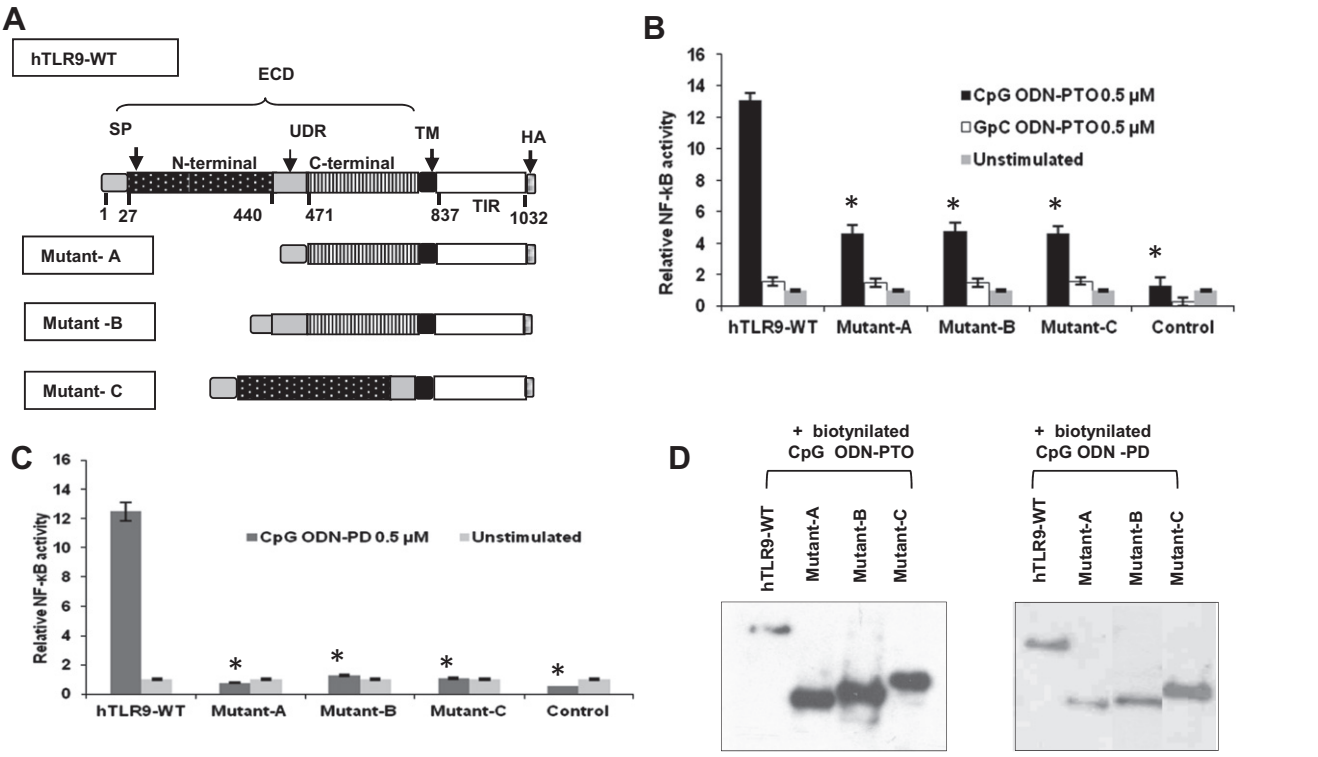

Fig. 1. Signaling activity and CpG ODN binding of truncation mutants of hTLR9. (A) Truncation scheme of hTLR9 mutants. SP is signaling peptide, TM is transmembrane domain, and TIR is Toll-Interleukin receptor domain. NF-κB activity of truncation mutants with CpG ODN–PTO (B) and CpG ODN–PD (C) stimulation. HEK-293-xl-null cells were used as a control. The asterisk (∗) denotes $p < 0.05$ showed significance compare to hTLR9-WT relative activity. The relative NF-κB activity is the luciferase activity of the ligand–stimulated receptor divided by the luciferase activity of the unstimulated receptor. (D) Cell lysates of HEK293-xl-expressing hTLR9-WT and mutants were immunoprecipitated with anti-HA. Eluates fraction treated with biotynilated CpG and recovered using Streptavidin magnetic bead.

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 [22,23]. 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. Mutant-C contains the N-terminal region. These mutants lost the signaling activity. Furthermore, stimulation with non-immunostimulatory GpC ODN–PTO that is inversion of the CpG to GpC totally abolished the signaling activity of the mutants (Fig. 1B). No significant difference in the expression level and localization was observed between hTLR9-WT and the truncation mutants (Supplementary 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 CpG ODN–PD that we developed recently [20] and demonstrated that all of the truncation mutants totally lost their signaling activity with CpG ODN–PD stimulation (Fig. 1C). Both the N- and C-termini of hTLR9-ECD bound to CpG ODN–PD (Fig. 1D) as same as that to CpG ODN–PTO. These result indicate that no difference in the signaling activity and binding capacity with CpG ODN–PTO and CpG ODN–PD was observed in the truncation mutants.

3.2. H505, Q510, H530, 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 LRR15 to 18 of the C-terminal region of the hTLR9-ECD that are involved in the binding of CpG ODN have been predicted [21]. We performed alanine scans of these residues and examined the effects of the mutations on receptor signaling activity. Addition of CpG ODN–PTO or CpG ODN–PD disrupted the signaling activity in cells expressing eight site-directed TLR9 mutants (R481A, N483A, H505A, Q510A, H530A, K532A, Y554A, and Q557A) (Fig. 2A and B). Mutation of H505, Q510, H530, 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 CpG-sequence dependent, where 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. S2.A and B). Therefore, these amino acid residues are essential for signaling activity and likely form ligand recognition sites in hTLR9. This result supplies the information regarding the ligand recognition site in TLR9 that mediates receptor activation, as depicted in Fig. 2C (focusing on LRR15 to LRR18 in hTLR9-ECD) and Supplementary Fig. S2.C (whole hTLR9-ECD).

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

In HEK293-xl-hTLR9 cells, our findings suggested that there is no difference in the mechanism by which hTLR9 recognizes CpG

ODN–PTO and CpG ODN–PD. To verify this, we examined the effect of the backbone on hTLR9 expressing human primary cells, PBMCs and B-cells 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 two times higher than of the level induced by CpG ODN–PD. Surprisingly, IL-6 secretion was also induced by GpC ODN–PTO, but not GpC ODN–PD both in PBMCs and B-cells (Fig. 3A). The IL-6 level induced by GpC ODN–PTO was almost half of that induced by CpG ODN–PTO. To confirm whether IL-6 secretion induced by GpC ODN–PTO is mediated by hTLR9, we utilized TLR9 specific inhibitory sequence 869 (IRS 869) simultaneously with both CpG and GpC ODN [24,25]. We confirmed that IRS 869 inhibits hTLR9 signaling with both CpG ODN–PTO and CpG ODN–PD (Fig. 3B). As we predicted, IRS 869 also inhibited IL-6 secretion from PBMCs stimulated by GpC ODN–PTO (Fig. 3B). 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 TLR9-depencence and sequence-independent manner.

4. Discussion

Although hTLR9 is highly similar to mTLR9 with 75% sequence homology, each of these proteins poses specific characteristics. hTLR9 recognizes a different CpG sequence from the sequence recognized by mTLR9. The optimal CpG sequence for mTLR9 ligand is an unmethylated CG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines. On the other hand, human PBMCs are strongly activated by CpG ODNs containing GTCGTT as the core motif [26]. In addition, proteolytic cleavage has been reported to transform mTLR9 into truncated C-terminal functional form [22,23,27]. However, to our knowledge, this truncation is less frequently reported in hTLR9. In this study, we observed truncated mutants of hTLR9 totally lost their responsiveness to both CpG ODN–PTO and 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. Recently, Qi et al. reported regulation of TLR3 stability and endosomal localization regulated by proteolytic-cleavage, but not the signaling activity [28]. Since TLR3 is member of nucleotide-sensing TLRs that predicted to share similar structure with TLR9, the truncation properties of TLR 9 is likely to be similar to that of TLR3.

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-terminal of hTLR9-ECD [13–15]. In contrast, only the D535/Y537 double mutant is reported to be involved in TLR9 signaling in the C-terminal of hTLR9-ECD [29]. Our study showed the pivotal role of eight amino acids in the C-terminal of hTLR9-ECD. These residues were distributed equally, with two residues each in LRR 15, 16, 17, and 18 of hTLR9. Replacing H505, Q510, H530, and Y554 with alanine was sufficient to negate hTLR9 stimulation, indicating that an interaction between these amino acids with CpG ODN is essential for hTLR9 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 vicinal-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.

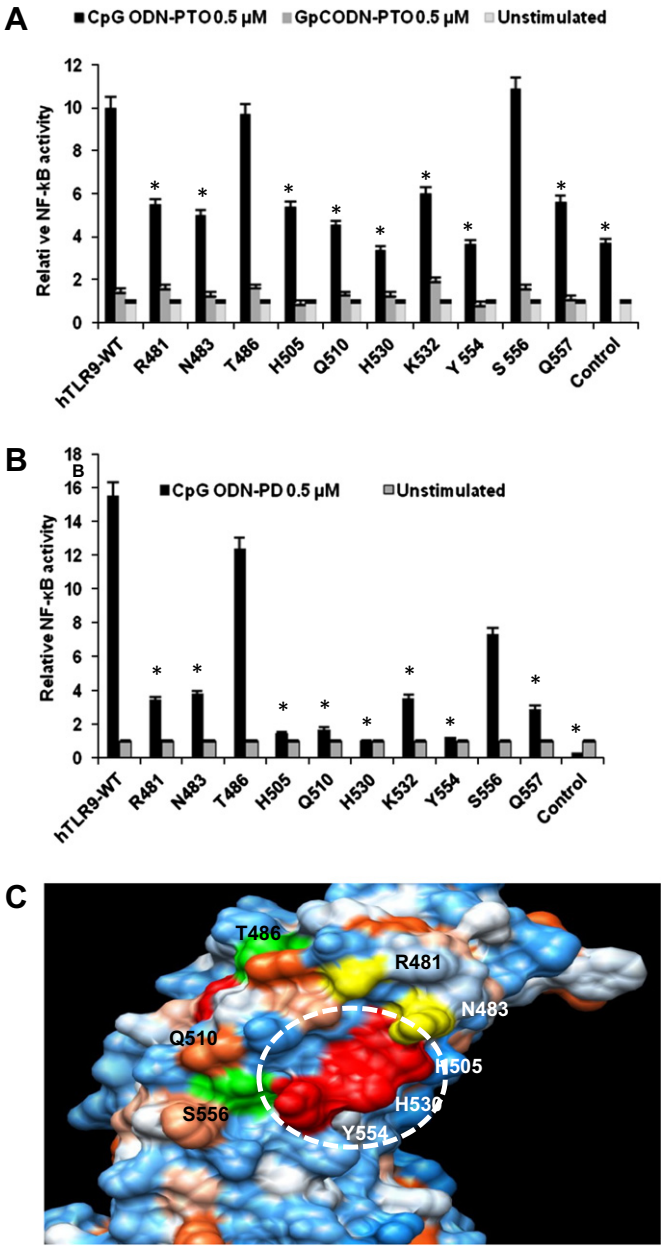

Fig. 2. Critical sites of signaling activity in the LRR 15 to 18 of hTLR9-ECD. NF-κB activity of mutants of predicted critical sites, stimulated with CpG ODN–PTO (A) and CpG ODN–PD (B). HEK-293-xl-null cells were used as a control. The asterisk (∗) denotes $p < 0.05$ showed significance compare to hTLR9-WT relative activity. (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]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CpG ODN–PTO is widely utilized as a TLR9 ligand because PTO backbone provides an high degree of nuclease-resistance. In spite of that, CpG ODN–PTO is also assumed to induce a non-specific reaction that causes antibody production and safety concerns [30]. In this study, we detected different activation signal from

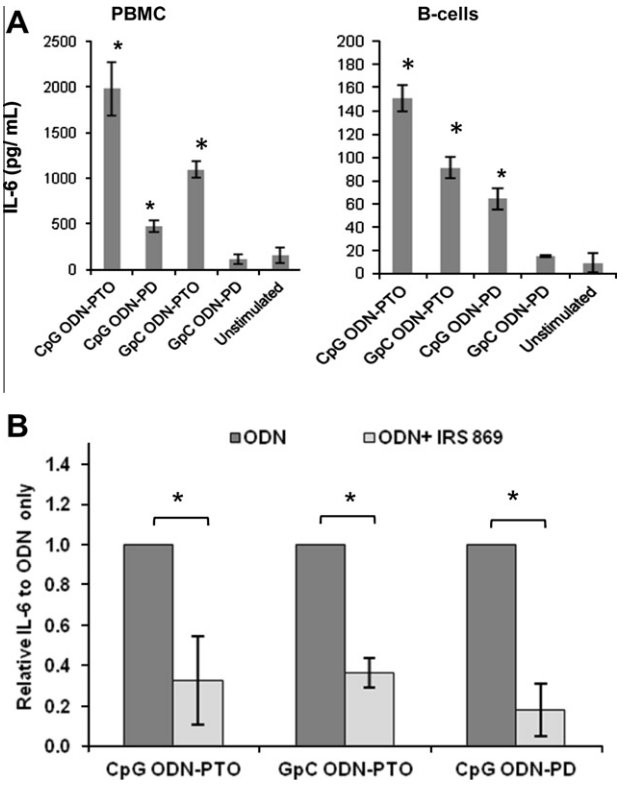

Fig. 3. IL-6 secretion in PBMCs and B cells induced by ODN–PTO and ODN–PD. PBMCs and B-cells (A) were incubated with immunostimulatory CpG and non-immunostimulatory GpC ODN. (B) PBMCs were incubated with CpG and GpC ODN in addition of IRS 869. PBMC stimulated with 0.5 μM ODN with or without 0.5 μM IRS 869 simultaneously. Relative IL-6 secretion level defined as IL-6 secretion level with any ODN stimulation and IRS 869 compare to with ODN stimulation only and incubated for 48 h before the measurement. The supernatants were harvested and IL-6 was measured by ELISA. The graphs represent mean and standard error. The asterisk (∗) denotes $p < 0.05$ showed significance compare to unstimulated. Experiments were performed in triplicate using three individual experiments (for human PBMCs) and in triplicate using two individual experiments (for $CD20^+$ B-cells).

our CpG ODN–PD and CpG ODN–PTO. In HEK293-xl-null cells, both CpG ODN–PTO and CpG ODN–PD activate hTLR9-WT, however CpG ODN–PTO induces high levels of NF-κB activity on all TLR9 mutants compared to CpG ODN–PD. This higher level activation is assumed associated with TLR9 conformational state that depend on ODN backbone. ODN–PD ligand contributes to the dimerization state of TLR9, whereas ODN–PTO 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. This higher activation of hTLR9 by CpG ODN–PTO stimulation might lead to biased interpretation to specify which mutants are highly essential for TLR9 signaling activity compared to by CpG ODN–PD. In PBMCs and B cells, we found that 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. We confirmed the IL-6 secretion triggered by not only CpG ODN–PTO but also GpC ODN–PTO sequence in PBMCs and B-cells. Other groups also showed that the PTO backbone allowed IL-6 secretion by PBMCs independently of CpG motif [16,31,32]. Several other reports suggest that CpG ODN–PTO can activate B cells in a CpG-sequence-independent manner [17,33–35]. 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 contrast, CpG ODN–PD induced IL-6 secretion in PBMCs and B-cell, but GpC ODN–PD did not, suggesting that CpG ODN–PD mediate innate immune response in a TLR9-dependent and in a CpG-sequence-dependent manner.

The goal of our study was to better understand the activation of human toll-like receptor TLR9 by CpG ODN. Using site-directed mutagenesis, we were able identify three residues in the C-terminus of the extracellular domain of TLR9 that are required for ligand activation. Further characterization of the function and three-dimensional structure of TLR9 will allow for the development of potential therapeutic agents. Additionally, our CpG ODN2006x3-PD displays specific recognition in TLR9; therefore, it could be a useful ligand for analyzing the ligand recognition and signaling mechanism of hTLR9.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.068.

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