Leucine-rich Repeat 11 of Toll-like Receptor 9 Can Tightly Bind to CpG-containing Oligodeoxynucleotides, and the Positively Charged Residues Are Critical for the High Affinity

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**Background:** Leucine-rich repeats (LRR) within the extracellular domain of human TLR9 (hTLR9) mediate binding to CpG ODN.

**Results:** LRR11 of hTLR9 has high affinity for CpG ODN, whereas the mutants of five positively charge residues in LRR11 lack this affinity.

**Conclusion:** LRR11 binds to CpG ODN with high affinity.

**Significance:** LRR11 could be further investigated as an antagonist of hTLR9.

TLR9 is a receptor for sensing bacterial DNA/CpG-containing oligonucleotides (CpG ODN). The extracellular domain (ECD) of human TLR9 (hTLR9) is composed of 25 leucine-rich repeats (LRR) contributing to the binding of CpG ODN. Herein, we showed that among LRR2, -5, -8, and -11, LRR11 of hTLR9 had the highest affinity for CpG ODN followed by LRR2 and -5, whereas LRR8 had almost no affinity. In vitro, preincubation with LRR11 more significantly decreased CpG ODN internalization, subsequent NF-κB activation, and cytokine release than with LRR2 and -5 in mouse peritoneal macrophages treated with CpG ODN. The LRR11 deletion mutant of hTLR9 conferred decreased cellular responses to CpG ODN. Single- or multisite mutants at five positively charged residues of LRR11 (LRR11m1–9), especially Arg-337 and Lys-367, were shown to contribute to hTLR9 binding of CpG ODN. LRR11m1–9 showed reduced inhibition of CpG ODN internalization and CpG ODN/TLR9 signaling, supporting the above findings. Prediction of whole hTLR9 ECD-CpG ODN interactions revealed that Arg-337 and Lys-338 directly contact CpG ODN through hydrogen bonding, whereas Lys-347, Arg-348, and His-353 contribute to stabilizing the shape of the ligand binding region. These findings suggested that although all five positively charged residues within LRR11 contributed to its high affinity, only Arg-337 and Lys-338 directly interacted with CpG ODN. In conclusion, the results suggested that LRR11 could strongly bind to CpG ODN, whereas mutations at the five positively charged residues reduced this high affinity. LRR11 may be further investigated as an antagonist of hTLR9.

Toll-like receptors (TLRs) are a family of pattern-recognition receptors able to distinguish pathogen-associated molecular patterns from pathogen constituents and then rapidly activate the innate and adaptive immune systems in response to infection (1). Overactivation or dysfunctional recognition will lead to inflammation or autoimmune disease. TLR9 is a receptor for sensing bacterial DNA/CpG-containing oligodeoxynucleotides (CpG ODN) within the endosomal compartment (2). Internalizing CpG ODN within the endosome initiates TLR9-mediated signaling via sequential recruitment of MyD88, interleukin receptor-associated kinase, and TNF receptor-associated factor 6 (TRAF6), which in turn activate important downstream transcription factors such as NF-κB and AP-1 that culminate in the induction of inflammatory cytokines such as TNF-α, IL-6, IL-1β, and IL-12 (3).

As a type I integral membrane glycoprotein, TLR9 consists of a pathogen binding extracellular domain (ECD) and cytoplasmic Toll/interleukin-1 receptor domain (4) joined by a single transmembrane helix (5). There are 25 leucine-rich repeats (LRRs) in the ECD of hTLR9, five (LRR2, -5, -8, -11, and -20) with inserted sequences predicted to bind to CpG ODN. Previously, LRR2, -5, and -8 bearing long insertions at position 10 after the consensus Asn residue were thought to bind to TLR9 (5). LRR8 contains a six-residue insert similar to a motif in a CpG binding protein that directly binds to unmethylated CpG dinucleotide sequences (6).

It was proposed recently that cleavage of the ECD within mouse TLR9 (mTLR9) occurs and that the fragment starting...
from LRR15 mediates ligand recognition (7, 8). The latest report finds that LRR2, -5, and -8 contribute to CpG ODN-mediated receptor activation, as deletions of these three LRRs lead to loss of mTLR9 binding (9). Another recent finding indicates that two variants of hTLR9, P99L within LRR2 and M400I within LRR13, are associated with altered receptor function in terms of NF-κB activation and cytokine induction (10), suggesting that the N-terminal fragment before LRR14 also binds to CpG ODN. The aim of this study was to identify and characterize the LRR region within hTLR9, which binds to CpG ODN with higher affinity and further determines which sites are critical for ligand binding.

**EXPERIMENTAL PROCEDURES**

*CpG ODN and Peptide Synthesis—*CpG ODN 1826 (CpG ODN, 5′-TCCATGCAGTCCTGGACGT-3′, the optimal immunostimulatory murine sequence), 5′-biotin-labeled CpG ODN 1826, and 6-FAM fluorescein-labeled CpG ODN 1826 with a nuclease-resistant phosphorothioate backbone were synthesized (Invitrogen). All peptides were synthesized by SBS Gene Technology, Ltd. (Beijing, China). Purities of the peptides were greater than 90%. All CpG ODNs and peptides were determined to be endotoxin-free using the LAL kit and an ATI 320–06 Kinetic Tube Reader (Lab Kinetics Ltd., Bruton, UK).

**HEK293T Cells and Isolation of Murine Peritoneal Macrophages—**The HEK293T cell line was purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM (high glucose) supplemented with 10% endotoxin-free fetal calf serum (FCS, Invitrogen). To obtain peritoneal macrophages, an equal number of male- and female-specific pathogen-free Kunming (Kunming) mice weighing 20–25 g were used. The mice were purchased from the Experimental Animal Center of the Third Military Medical University (Chongqing, China). All animal experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals. The murine peritoneal macrophages were isolated as described previously (11, 12) and cultured in RPMI 1640 supplemented with 10% endotoxin-free FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin in a 37 °C humidified atmosphere with 5% CO2. Live cells diluted with 0.4% trypan blue in phosphate-buffered saline (PBS, 0.1 mM, pH 7.2–7.4) were counted using a hemacytometer.

**Quantitative Affinity Assay of Peptides for CpG ODN—**The first type of affinity assay was conducted using an IAsys Plus affinity biosensor (Thermo Labystem, Altrincham, Cheshire, UK) according to a previous report (13). Biotinylated CpG ODN was immobilized on the surface of a biotin-coated cuvette as described previously. Peptides (50 μM) were added into the cuvette and allowed to bind for 3 min. A single binding curve of each peptide was generated. After the cuvette was washed 3 times with 50 μl of PBS and alternately washed twice with 0.01 M HCl, the data were collected and analyzed using FASTplot (Thermo Labystem, Altrincham, Cheshire, UK).

The second type of quantitative affinity assay was conducted using a dual-polarization interferometry (DPI) method according to a previous report (14). The CpG ODN was immobilized on the surface of a sample cuvette on an AnaLight Bio200 DPI biosensor (Fairfield, Altrincham, Cheshire, UK). Gradient concentrations of peptides were added into the cuvette to detect the real-time mass changes of peptides, which specifically bind to CpG ODN. The dissociation equilibrium constant (K_d) value of each peptide was calculated using AnaLight Explorer software.

**Cytokines Release Assays—**Cells (4 × 10^5 cells) were incubated for 4 h, and the supernatants were then discarded and harvested for extracting nuclear proteins. TransAM NF-κB activation assays were performed as described previously (14). The cell cultures were then incubated with a 1:100 dilution of NF-κB p50 and p65 in nuclear extracts and were then incubated with secondary goat anti-mouse IgG antibody (Beyotime) and developed with the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Pierce, Rockford, IL) for chemiluminescence detection with a ChemiDoc XRS gel imaging system (Bio-Rad).

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replaced with fresh medium. CpG ODN, LPS, or Pam3 and peptides were preincubated for 15 min, and then the mixtures were added to the cultures. After incubation for 4, 6, and 8 h at 37 °C in a 5% CO₂-humidified incubator, the supernatants were collected for detecting TNF-α, IL-6, and IFN-γ using corresponding ELISA kits (R&D Systems, Minneapolis, MN).

Real-time PCR Analysis of mRNA Expression—Cells treated as described above were harvested for extracting total RNA using TRIzol reagent (Roche Applied Science). Total RNA samples were reverse-transcribed using the Primerscript RT reagent kit (Takara, Dalian, Liaoning, China), and the cDNA samples were used as templates to detect mRNA expression of TNF-α, IL-6, and IFN-γ by real-time PCR using primers listed in supplemental Table S1. Real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad) with previously described reaction conditions (17). After the PCR was finished, dissociation curve analysis was performed to detect amplification of any nonspecific products. For each sample, mRNA expression levels for specific transcripts were normalized to the amount of corresponding ELISA kits (R&D Systems, Minneapolis, MN).

Generation of the TLR9 Deletion Mutant, HEK293T Transduction, and Activation Assays—The hTLR9 gene was amplified from the pcDNA3-hTLR9 plasmid (maintained in our laboratory) and subcloned into the lentiviral vector GV186 (Genechem, Shanghai, China). The LRR11-deleted hTLR9 mutant was generated using primers containing the desired mutations with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). After sequencing, the hTLR9-WT and hTLR9-del_LRR11 plasmids were used for packaging into lentiviruses. HEK293T cells (2 × 10⁵ cells) were cultured in 6-well plates until 30% confluency and then transduced with the lentiviruses expressing hTLR9 (2 × 10⁶ Transducing unit/ml) for 72 h. After resistance screening for three generations, the transduced cells were used to detect TLR9 mRNA expression and for immunoblot assays using an anti-TLR9 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). IκB-α degradation, NF-κB activation, and TNF-α release were tested as described above in hTLR9-WT- or hTLR9-del_LRR11-expressing HEK293T cells (5 × 10⁶, 1 × 10⁷, and 5 × 10⁶ cells) after treatment with CpG ODN (1.5 μM).

Molecular Modeling—Because the crystal structure of hTLR9 ECD or hTLR9 ECD in complex with CpG ODN has not been solved, we generated a computed three-dimensional structural model of the hTLR9 ECD-CpG ODN complex. First, the structures of the hTLR9 ECD and the CpG ODN substrate were predicted by a homology modeling approach. The CpG ODN model was then docked onto the hTLR9 ECD model. Searches for reference proteins, sequence alignments, and homology modeling were performed in Discovery Studio 2.5 (Accelrys Inc. San Diego, CA). To improve the alignment of the target and templates, profile-profile alignment was performed (19). The three-dimensional structure of hTLR9 ECD was created using the MODELLER program in Discovery Studio 2.5 (20). Quality assessment of the predicted structure was evaluated using the Verify Protein (Profiles-3D) protocol. Surface charge calculations were conducted with the PyMol visualization software using PDB2PQR (21), PropKa (22), and APBS packages (23).

Biopolymer tools in Discovery Studio 2.5 were employed to build the CpG ODN model. Because the immune activity of CpG ODN depends on a single-stranded form (24), the CpG ODN structure was modeled as a single-stranded CpG ODN (ssCpG ODN) molecule. The ssCpG ODN molecule was generated in the B-form using standard helix parameters. The program Hex 6.1 was used to perform a rigid-body docking search (25). In the docking calculations of the Hex program, hTLR9 ECD was defined as the receptor, and CpG ODN was defined as the ligand. The shape plus electrostatic correlation algorithms were used. Other parameters used for the docking processes were set at default values.

Statistics and Presentation of Data—Cytokine concentrations and other data were expressed as the means ± S.D. Fifty percent inhibitory concentration (IC₅₀) values were calculated with Origin 7.0 using the sigmoidal fit method. Student’s t test was used for paired comparisons, and the one-way analysis of variance test with post hoc Bonferroni correction was used for multiple comparisons. Differences with p < 0.05 were considered statistically significant, and those with p < 0.01 were considered highly statistically significant.

RESULTS

LRR11 Binds to CpG ODN with the Highest Affinity among LRR2, -5, -8, and -11

LRR11 Possesses High Affinity for CpG ODN—If LRR11 is responsible for binding CpG ODN, it should have higher affinity for this substrate than other regions within hTLR9. Therefore, LRR2, -5, -8, and -11 peptides and an unrelated peptide (URP) were synthesized (sequences shown in Fig. 1A) to test the above hypothesis. Using the IAsys biosensor, the LRR11 peptide was confirmed to have the highest binding affinity for CpG ODN, whereas the URP showed no binding ability; meanwhile, LRR2 and -5 exhibited lower binding affinity for CpG ODN, and LRR8 had almost no binding affinity (Fig. 1B). Similar results were observed using the DPI biosensor. The Kᵦᵦ values of LRR11 and LRR2 for CpG ODN were 4.79 nM and 6.95 μM (Fig. 1, C1 and C2), respectively, suggesting that LRR11 had a very high affinity for CpG ODN. Meanwhile the Kᵦᵦ values of LRR5 and -8 could not be detected due to low affinity (data not shown). Using CD spectroscopy, the structural changes in CpG ODN upon titration with peptides were observed. As expected, the addition of LRR11 to CpG ODN resulted in significant changes in the CD spectra (Fig. 1D1), suggesting that LRR11 interacted with CpG ODN with high affinity. Meanwhile, the addition of LRR2 resulted in less change in the CD spectra (Fig. 1D2), and LRR5 and -8 did not cause any changes (data not shown), suggesting these three peptides had very low or no affinity for CpG ODN. The above results from three independent experiments demonstrated that LRR11 had the highest binding affinity for CpG ODN compared with the relatively lower affinity of LRR2 and -5, suggesting that LRR11 was the main region of hTLR9 binding to CpG ODN.
LRR11 Most Significantly Decreases CpG ODN Internalization within Macrophages—Internalization of CpG ODN is a prerequisite for CpG ODN-induced cell activation. If either LRR2, -5, -8, or -11 is the main region for binding to CpG ODN, preincubation of CpG ODN with the peptide would lead to decreased internalization and subsequent accumulation of CpG ODN within the macrophages. As expected, using confocal microscopy, the LRR11 peptide was found to most significantly decrease the fluorescence intensity produced by 6-FAM CpG ODN within mouse macrophages (Fig. 2A). LRR2 and -5 also decreased the fluorescence intensity produced by 6-FAM CpG ODN, but the effects were much lower than that observed with LRR11. Meanwhile, LRR8 had no influence on the fluorescence intensity, which was similar to the result with URP. Additionally, LRR11 decreased CpG ODN internalization in a dose-dependent manner (Fig. 2B). Non-labeled CpG ODN could competitively bind LRR11, leading to increased free molecules of 6-FAM CpG ODN and a subsequent increase in fluorescence intensity within the cells as well (Fig. 2C). These results were consistent with those from the affinity assay above, suggesting that LRR11 was the main region of hTLR9 binding to CpG ODN.

LRR11 Most Significantly Decreases IκBα Degradation and NFκB Activation within Macrophages—Activated TLR9-mediated signaling leads to rapid IκBα degradation and subsequent NF-κB activation (4). Because internalization of CpG ODN was decreased by preincubation with LRR11, subsequent IκBα degradation and NF-κB activation were also presumed to decrease. As expected, CpG ODN was found to induce IκBα degradation and activation of the NF-κB p50 and p65 subunits, whereas preincubation with LRR11 significantly decreased IκBα degradation and NF-κB activation within cells treated with CpG ODN. Although LRR2 and -5 also decreased IκBα degradation and NF-κB activation, their effects were much lower than those observed with LRR11, and LRR8 showed no such influence (Fig. 3A). Meanwhile, preincubation with LRR11 did not affect IκBα degradation and NF-κB activation within cells treated with LPS (the ligand of TLR4) or PAM3 (the ligand of TLR2) (Fig. 3B), demonstrating that LRR11 only specifically bound to CpG ODN and not to LPS or PAM3. The LRR11 peptide
decreased CpG ODN-mediated NF-κB activation in a dose-dependent manner (Fig. 3B), and the IC₅₀ was 0.84 μM for the p50 subunit and 0.53 μM for the p65 subunit. These results were consistent with those from the experiments above, further supporting that LRR11 was the main region of hTLR9 binding to CpG ODN.
LRR11 Most Significantly Decreases mRNA Expression of Cytokines and Their Release from Macrophages—The release of cytokines such as TNF-α, IL-6, and IFN-γ is an indicator of CpG ODN/TLR9-mediated macrophage activation (26). Because the LRR11 peptide could decrease NF-κB activation, preincubation with LRR11 may also decrease the mRNA expression of cytokines and their release from macrophages induced by CpG ODN. As expected, none of the LRR peptides alone affected cytokine release (supplemental Fig. S1), whereas CpG ODN could increase the release of TNF-α, IL-6, and IFN-γ (Fig. 4A). The effects of LRR11 on decreasing the mRNA expression and release of these cytokines were most significant, whereas those of LRR2 and -5 were much lower, and LRR8 expectedly had no such influence (Fig. 4A). Meanwhile, preincubation with LRR11 did not affect the mRNA expression and release of the cytokines induced by LPS or PAM3 (Fig. 4B), demonstrating that LRR11 only specifically bound to CpG ODN and not LPS or PAM3 to inhibit cytokine release. The LRR11 peptide decreased the mRNA expression and release of these cytokines in a dose-dependent manner (Fig. 4C), and the I_{50} of the LRR11 peptide was 19.26 μM for TNF-α, 1.89 μM for IL-6, and 0.72 μM for IFN-γ. These results were consistent with those from the experiments above, demonstrating that LRR11 was the main region of hTLR9 binding to CpG ODN.

LRR11 Deletion Mutant of hTLR9 Confers Decreased Responses to CpG ODN—To confirm LRR11 binding to CpG ODN, the plasmid expressing the LRR11-deleted hTLR9 mutant was transduced into HEK293T cells. If LRR11 is the main region for hTLR9 binding to CpG ODN, its deletion would at least partly disrupt the cellular responsiveness to CpG ODN. As expected, HEK293T cells transduced with the TLR9-WT plasmid responded well to CpG ODN, whereas those expressing TLR9-del_LRR11 partially lost the responsiveness to CpG ODN; activation of NF-κB p50 and p65 and mRNA expression and release of TNF-α decreased by about 61, 50, 72, and 57%, respectively (Fig. 5). These results were consistent with those from the experiments above, confirming that LRR11 was the main region of hTLR9 binding to CpG ODN.

Positively Charged Residues within LRR11 Are Critical for High Affinity

LRR11m1–9 Have Very Low Affinity for CpG ODN Compared with LRR11—Knowing that LRR11 was the main region for binding to CpG ODN, we sought to evaluate the critical residues critical for ligand binding. The sequence analysis revealed five positively charged residues (Fig. 6A) and two distinct positively charged patches (Fig. 6B) within LRR11. Mutations of the five positive residues (R337S, K338N, K347N, R348S, and H353Q) obviously changed the two patches (Fig. 6B). Therefore, peptides with single- or multiple-site mutations in the positively charged residues of LRR11 (LRR11m1–9) were designed (Fig. 6A), and four single-site mutations in the uncharged residues (LRR11m10–12) were designed as unrelated-control peptides (sequences shown in Fig. 6A). LRR11m1, -2, -3, -4, and -5 each possessed only a single mutation. LRR11m6 and -7 each possessed two mutations (R337S and K338N, K347N and R348S, respectively). LRR11m8 contained four mutations (R337S, K338N, K347N, and R348S), and LRR11m9 had all five mutations. As expected, the single-site mutant LRR11m1–5 lost part of the binding to CpG ODN compared with the high affinity of LRR11 for CpG ODN. The mutants in order of having the greatest to least affinity loss were R337S, K338N, H353Q, R348S, and K347N, suggesting the relative contribution of the parent residue to the high affinity of LRR11. The double-site mutants LRR11m6 and LRR11m7 had lower binding affinity than their corresponding single-site mutants. However, LRR11m6 had much lower affinity than LRR11m7, suggesting Arg-337 and Lys-338 contributed more to the high affinity of LRR11. Consistently, the four-site mutant LRR11m8 nearly lost all affinity. Significantly, the five-site mutant LRR11m9 lost all binding affinity (Fig. 6C). The results suggested all five of the positively charge residues were involved in binding of LRR11 to CpG ODN, whereas Arg-337 and Lys-338 may have had greater contributions to the high affinity of LRR11.

LRR11m1–9 Have Lower Capacity Compared with LRR11 to Prevent CpG ODN Internalization by Macrophages—Considering LRR11m1–9 showed reduced or lost affinity for CpG ODN, we hypothesized that more of the CpG ODN molecules preincubated with the mutant LRR11 would enter macrophages than those preincubated with the parent LRR11. In other words, due to LRR11m1–9 binding to fewer CpG ODN molecules, more CpG ODN would be internalized and accumulated within macrophages (the lower the affinity, the more CpG ODN molecules entering the cells). As expected, the results of the internalization assay showed that LRR11m1–9 resulted in an increase in CpG ODN accumulation compared with LRR11. Compared with LRR11, preincubation with LRR11m6, -8, and -9 led to the highest increase (i.e. low inhibitory effect), and LRR11m3, -4, and -5 led to the lowest increase (i.e. high inhibitory effect), and LRR11m3, -4, and -5 led to the lowest increase (i.e. high inhibitory effect).


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**A**

**B**

**C**

LRR11m1–9 Have Reduced Abilities Compared with LRR11 to Inhibit CpG ODN-mediated IkBα Degradation and NF-κB Activation—Theoretically, preincubation of the LRR11m1–9 (having lost or reduced affinity) with CpG ODN would lead to less inhibition of IkBα degradation and NF-κB activation than preincubation with the parent LRR11. As expected, preincubation of LRR11m1–9 resulted in increased IkBα degradation and NF-κB activation compared with preincubation with LRR11; LRR11m6, -8, and -9 led to the highest increase, and LRR11m3,
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-4, and -5 led to the lowest increase (Fig. 8). The results were also in line with those of the affinity assay and CpG ODN internalization experiment, again suggesting the five positively charged residues within the LRR11 were essential for the high affinity of LRR11, but Arg-337 and Lys-338 may have contributed more than the other residues to the binding.

LRR11m1–9 Have Reduced Ability Compared with LRR11 to Inhibit CpG ODN-mediated mRNA Expressions and Their Release of Cytokines—Theoretically, LRR11m1–9 with reduced or lost affinity for CpG ODN would have reduced abilities, as compared with LRR11, to inhibit CpG ODN-induced NF-κB activation and subsequent release of cytokines from macrophages. Herein, none of the LRR11m1–9 peptides alone could induce cytokine release (supplemental Fig. S1), but preincubation of CpG ODN with LRR11m1–9 indeed resulted in increased mRNA expression and release of TNF-α, IL-6 and IFN-γ from the cells compared with the condition in which CpG ODN was preincubated with LRR11 (Fig. 9). As expected, mutations of uncharged sites could not increase cytokine release compared with wild-type LRR11 (supplemental Fig. S3). These findings were also in line with the results of the affinity assay, CpG ODN internalization, IκBα degradation, and NF-κB activation experiments, again suggesting that the five positively charged residues within LRR11 were essential for its high affinity, with Arg-337 and Lys-338 being more important than the other residues.

Positively Charged Residues within LRR11 Participate in hTLR9 ECD-CpG ODN Interactions—To investigate how the positively charged residues interact with CpG ODN, the three-dimensional structure of hTLR9 ECD was constructed using a homology modeling strategy. PDB BLAST results indicated that the crystal structures of the ECD within human TLR2, -3, and -4 were suitable templates for constructing an hTLR9 ECD model. The compatibility of the hTLR9 ECD model with the amino acid sequence was
assessed, and the Verify Score was 275.1, higher than the Verify Expected Low Score value (164.1), indicating the model was acceptable.

The default protein-DNA docking protocol implemented in Hex Version 6.1 was used for the docking runs. The final complex model was characterized in terms of the interaction features to improve our understanding of the mechanism of hTLR9-CpG ODN interaction. The interaction pattern showed that the hTLR9 ECD-CpG ODN interaction sites are located close to the central region of the hTLR9 ECD (Fig. 10A). In the model the interface between the receptor and CpG ODN molecule is geometrically complementary. Because of the considerable size of CpG ODN, the ligand binding region contains many interacting residues. Residues within contact distance of CpG ODN include Tyr-224, Val-248, Glu-287, Leu-289, Val-290, Arg-311, Ser-316, Thr-334, Gln-335, Leu-336, Leu-337, Lys-338, Asn-340, Lys-367, Met-392, Arg-397, and Trp-447. These residues form a large contact area for binding to the CpG ODN molecule. In the binding region, residues Glu-287, Arg-311, Val-312, Ser-316, Thr-334, Leu-336, Arg-337, Lys-367, Asn-340, Arg-397, and Trp-447, mostly located on the concave face of the horseshoe, are apparently important for ligand binding as they make direct and close contact with the CpG ODN molecule.

![Figure 5](image-url)

**FIGURE 5.** IκB-α degradation, mRNA expression, and release of TNF-α from HEK293T cells expressing TLR9-WT or TLR9-del_LRR11. A, hTLR9 expression in the transduced HEK293T cells was tested by Western blotting. B, IκB-α degradation and NF-κB activation in the transduced HEK293T cells treated with or without CpG ODN (1.5 μM) were tested using Western blotting. **, p < 0.01 versus TLR9-WT. Data from one of two independent experiments (n = 3) are shown (mean ± S.D.). C, mRNA expression and release of TNF-α from transduced HEK293T cells treated without or with CpG ODN (1.5 μM) were tested using real-time PCR and ELISA, respectively. **, p < 0.01 versus TLR9-WT. One of two independent experiments (n = 3) is shown (mean ± S.D.).
affinity for CpG ODN, whereas Arg-337 and Lys-338 were most important. In the close-up view of the binding region within the predicted hTLR9 ECD-CpG ODN model (Fig. 10A), hTLR9 ECD is anchored with the CpG ODN strand at Arg-337 and Lys-367, highly consistent with our experimental data, whereas Lys-347, Arg-348, and His-353 do not directly make contact with CpG ODN. The predicted complex model suggested that hydrogen bonding and van der Waals contacts between receptor residues and CpG ODN play key roles in hTLR9 ECD-CpG ODN interactions. The detailed computed molecular interactions indicated that Arg-337-CpG ODN and Lys-338-CpG ODN contribute mainly hydrogen bonds in the complex (Fig. 10B). Although Lys-347, Arg-348, and His-353 do not make direct contact with the ligand, they contribute to stabilizing the shape of the ligand binding region via connecting the β-sheet of the convex surface.

DISCUSSION
To identify the specific LRR within the ECD of hTLR9 responsible for binding to CpG ODN and the key residues contributing to this ligand binding, a unique approach was established based on an affinity binding assay, several competitive binding assays, and computed molecular docking models. Herein, we reported that the LRR11 peptide could bind to CpG ODN with high affinity, significantly decreasing CpG ODN internalization and subsequent CpG ODN/TLR9 signaling. The hTLR9 mutant with a deletion in LRR11 conferred decreased responses of HEK293T cells to CpG ODN. Mutant peptides with single- or multiple-site alterations among the five residues had decreased binding affinity to CpG ODN, with Arg-337 and Lys-338 contributing most to the high affinity.

Based on LRR8 with a six-residue insert similar to a motif identified in the CpG binding protein that directly binds to unmethylated CpG dinucleotide sequences (6), Bell and Mullen (5) suggested that LRR2, -5, and -8 bearing long insertions at position 10 after the consensus Asn residue may be the regions recognizing and binding to CpG ODN. In 2008, Ewald et al. (7) reported that, although both the full-length and cleaved forms...
of mTLR9 are capable of binding ligand, only the processed form can recruit MyD88, indicating the truncated receptor rather than the full-length form is functional. Based on the observed molecular mass, they estimated that cleavage occurs within or distal to LRR14. In 2009, Peter et al. (9) showed that by individual deletion of LRR2, -5, or -8, but not of LRR11 or -20, the functional integrity of the mTLR9 ECD to inhibit CpG ODN binding and receptor activation is lost. They further identified a positively charged region in the N terminus that is essential for CpG ODN-induced mTLR9 activation (9). This interaction site mirrored the previous finding for the structural recognition of dsRNA by TLR3 and hinted toward a general principle of nucleic acid recognition by the respective TLR (27). In this study, LRR2, -5, -8, and -11 peptides were synthesized, but not the LRR20 peptide, as it was reported to have no contribution for TLR9 binding to CpG ODN (9).

The $K_D$ value is the gold standard measurement for molecular interactions. To correctly represent the molecular interactions of the LRR2, -5, -8, or -11 peptide with CpG ODN, synthesized CpG ODN was immobilized on the surface of the DPI biosensor, a widely used label-free method for the direct detection of interaction between immobilized molecules and sampling substances (14). LRR11 was found to possess the highest affinity for CpG ODN, with a $K_D$ value of 4.79 nM. Meanwhile, the $K_D$ values of LRR2, -5, and -8 for CpG ODN were very low or could not be detected, suggesting that LRR11 had the highest affinity for CpG ODN, unlike the previous findings (7, 9).

Internalization of CpG ODN is prerequisite for CpG ODN-induced cell activation, and subsequent NF-κB activation and release of cytokines such as TNF-α are characteristics of the CpG ODN/TLR9-mediated signaling pathway (26). Now that a specific LRR was identified as the sequence binding to CpG ODN, preincubation of CpG ODN with the peptide was hypothesized to lead to decreased internalization and accumulation of CpG ODN as well as subsequent decreased IκBα degradation, NF-κB activation, and cytokine release. As expected, the ability of CpG ODN preincubated with LRR11 to activate NF-κB and the downstream effects decreased most significantly compared with that observed with LRR2 and -5, whereas no change occurred with LRR8. These results confirmed that only LRR11 could bind to CpG ODN with high affinity. Meanwhile, LRR2 and -5 exhibited lower affinity, and LRR8 showed almost no binding to CpG ODN.

To confirm LRR11 as the main region for hTLR9 binding to CpG ODN, the hTLR9-del_LRR11 lentiviral plasmid (LRR11-deleted mutant) was transduced into HEK293T cells. As expected, HEK293T cells with the TLR9-WT plasmid responded well to CpG ODN and released high levels of TNF-α. In contrast, cells with TLR9-del_LRR11 partly lost the responsiveness to CpG ODN, confirming that LRR11 was the region of hTLR9 binding to CpG ODN. The question is why would only about 50–70% of the CpG ODN-mediated signaling be lost. From results of the assays determining affinity, CpG ODN internalization, IκBα degradation, NF-κB activation, and cytokines release, we found LRR2 and -5 showed some affinity for CpG ODN. We presumed that TLR9 could weakly bind to CpG ODN via LRR2 and -5 when LRR11 was completely deleted, leading to incomplete activation of the cells. Therefore, the TLR9 mutant with deletion of only LRR11 could still respond to CpG ODN. These results further indicated that LRR11 was the main region for TLR9 binding to CpG ODN, and LRR2 and -5 could also contribute to the binding, albeit at a much lower level than that of LRR11.
In 2004, Rutz et al. (24) suggested that TLR9 binds directly and specifically to an unmethylated ssCpG ODN sequence and identified a potential CpG ODN binding domain (LRR17) homologous to that described for methyl-CpG ODN binding proteins by sequence comparison. Amino acid substitutions in this region abrogated CpG ODN binding and led to loss of NF-κB activation. In 2008, Jin and Lee (3) thought that TLR9 as well as TLR3, -5, -7, and -8 belong to the single-domain subfamily that interacts with hydrophilic proteins or nucleic acids based on sequence and structure analysis. In 2008, Liu et al. (27) revealed several conserved histidine residues, including His-39 and His60, which directly bind to the negatively charged dsRNA based on a previously solved crystal structure of TLR3 in the N terminus (28). In 2009, Peter et al. (9) showed that surface charge is important for TLR9 activation by CpG ODN and that an N-terminal positively charged area is important for TLR9 activity. Mutating Lys-51 or Arg-74 to the uncharged amino acid methionine led to nearly complete loss of the receptor function. In our study, after bioinformatics analysis of the LRR11 sequence of hTLR9, we also considered that the positively charged residues may be important for LRR11 binding to CpG ODN, which has a similar structure and physicochemical properties to those of dsRNA. Our results showed that all five of the positively charge residues (Arg-337, Lys-367, Lys-347, Arg-
348, and His-353) in LRR11 contributed to ligand binding because their mutations (R337S, K338N, K347N, R348S, and H353Q) decreased the affinity of LRR11 for CpG ODN. Among the five single mutations, R337S and K338N led to much greater losses of the affinity of LRR11. Subsequent analyses of CpG ODN accumulation, NF-κB activation, and cytokine release were consistent with the results from the affinity assay.

To further analyze the binding region of LRR11, the whole hTLR9 ECD-CpG ODN complex was constructed using bioinformatics methods. The ssCpG ODN instead of dsCpG ODN was used to establish the hTLR9 ECD-CpG ODN complex based on a previous finding that dsCpG ODN interacts more weakly with TLR9 than ssCpG ODN (24). In 2003, Bell et al. (5) suggested that the ligand-binding site should be located on the concave surface of the LRR solenoid based on all LRR-ligand complexes solved at atomic resolution. Our results showed Arg-337 and Lys-367, which are localized on the concave surface of hTLR9, directly contact CpG ODN via hydrogen bonds. Meanwhile, Lys-347, Arg-348, and H353, which are located in the insertion loop of LRR11, do not directly come into contact with the ligand but do connect the β-sheet of the convex surface. Mutations of three of the residues could distort the charge distributions and the shape of the interface surface of hTLR9.

In conclusion, LRR11 of hTLR9 could tightly bind to CpG ODN, with all five of its positively charged residues, especially Arg-337 and Lys-367, contributing to the high affinity. LRR2 and -5 also contributed to the binding, but their contributions were much lower than that of LRR11. LRR11 should be further investigated as an antagonist of hTLR9 to decrease cellular activation induced by CpG ODN.

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