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Activation of rabbit TLR9 by different CpG-ODN optimized for mouse and human TLR9

Jin Liu[a,1,2], Congfeng Xu[a,1,3], Yi-Ling Liu[b], Hanako Matsuo[a], Rebecca Pe-feng Hsieh[a], Jeng-Fan Lo[c], Ping-Hui Tseng[d], Chiun-Jye Yuan[e], Yunping Luo[f], Rong Xiang[g], Tsung-Hsien Chuang[a,b,*]

[a] *Infectious and Inflammatory Disease Center, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, United States*
[b] *Immunology Research Center, National Health Research Institutes, Miaoli County, Taiwan*
[c] *Institute of Oral Biology, National Yang-Ming University, Taipei, Taiwan*
[d] *Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan*
[e] *Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*
[f] *Department of Immunology, School of Basic Medicine, Peking Union Medical College, Beijing, PR China*
[g] *School of Medicine, University of Nankai, Tianjin, PR China*

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ABSTRACT

Synthetic CpG-oligodeoxynucleotides (CpG-ODN) are potent adjuvants that accelerate and boost antigen-specific immune responses. Toll-like receptor 9 (TLR9) is the cellular receptor for these CpG-ODN. Previous studies have shown species-specific activation of mouse TLR9 (mTLR9) and human TLR9 (hTLR9) by their optimized CpG-ODN. The interaction between rabbit TLR9 (rabTLR9) and CpG-ODN, however, has not been previously investigated. Here, we cloned and characterized rabTLR9 and comparatively investigated the activation of the rabbit, mouse, and human TLR9 by CpG-ODN. The complete open reading frame of rabTLR9 encodes 1028 amino acid residues, which share 70.6% and 75.5% of the identities of mTLR9 and hTLR9, respectively. Rabbit TLR9 is preferentially expressed in immune cells rich tissues, and is localized in intracellular vesicles. While mTLR9 and hTLR9 displayed species-specific recognition of their optimized CpG-ODN, rabbit TLR9 was activated by these CpG-ODN without any preference. This result suggests that rabTLR9 has a broader ligand-recognition profile than mouse and human TLR9.

Abbreviations: TLR, toll-like receptor; CpG-ODN, CpG-oligodeoxynucleotides; NF-κB, nuclear factor-κB; MyD88, myeloid differentiation factor 88; IRAK, IL-1R associated-kinase; TRAF6, TNFR-activated factor 6; TAK1, TGF-β-activating kinase; HEK, human embryonic kidney; FCS, fetal calf serum; PBS, phosphate buffer saline; DAPI, 4′,6 diamidino-2-2 phenylindole; RACE, rapid amplification of cDNA end.

* Corresponding author at: Immunology Research Center, National Health Research Institutes, Zhunan, Miaoli County 35053, Taiwan. Tel.: +886 37 246166x37611.
E-mail address: thchuang@nhri.org.tw (T.-H. Chuang).

[1] These authors contributed equally to this work.

[2] Current address: Shanghai Veterinary Institute, Chinese Academy of Agricultural Sciences, Shanghai, PR China.

[3] Current addresses: Shanghai Institute of Immunology, Institutes of Medical Sciences, Shanghai Jiao Tong University School of Medicine, and Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institute of Biological Sciences, Chinese Academy of Science, Shanghai, PR China.

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1. Introduction

Toll-like receptors (TLRs) play a critical role in host defense to microbial infections by sensing a wide variety of microbial pathogens with diverse structures from lipids, lipoproteins, glycans, proteins to nucleic acids [1–5]. TLR9 belongs to a subfamily of these TLRs, comprising TLR3, TLR7, TLR8, and TLR9, that recognizes nucleic acids [5,6]. This subfamily of TLRs is distinct from other TLRs in their cellular localization. Whereas others are expressed on cell surface, these four TLRs are located in the intracellular vesicles [7,8].

Bacterial and viral DNA are potent stimuli to immune cells. This immunostimulatory activity is assigned to sequence motifs containing unmethylated CpG-dideoxynucleotides. Synthetic CpG-oligodeoxynucleotides (CpG-ODN) mimic the stimulatory effect of these microbial DNA in activation of immune cells. The activity of a CpG-ODN is determined by its length; the number of CpG motifs; and the spacing, position, and surrounding bases of these motifs. In addition, there are species-specific differences for these CpG-ODN. For example, CpG-ODN containing GTCGTT motif preferentially activates human cells, whereas CpG-ODN with GACGTT motif displays the greatest activity toward mouse cells [9–13].

TLR9 is the cellular receptor to mediate the function of these CpG-ODN [14–16]. Upon activation, this TLR recruits MyD88 to form a MyD88/IRAK1/IRAK4/TRAF6 complex. This in turn activates TAK1, leading to the activation of NF-κB and production of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-12. In plasmacytoid dendritic cells, TLR9 activates IRF7 through the MyD88/IRAK1/IRAK4/TRAF6 complex, leading to the production of type I IFNs [17,18]. Because of the potent immune responses facilitate eradication of viral infected cells and cancer cells from bodies, CpG-ODN are investigated for their therapeutic application for treatment of infectious diseases and cancers [19,20]. In addition, these CpG-ODN are being developed as adjuvant to boost antigen-specific immune responses. In mice, they have been shown to be a very strong adjuvant to promote Th1 type of immune response [21,22]. The complete Freund's adjuvant (CFA) is commonly used for induction of cell-mediated immune responses in rodents. Nevertheless, CpG-ODN performing better in induction of Th1 type immune response, and appear to have an advantage over CFA in that they do not cause the severe local inflammation associated with the CFA [21,23,24].

Rabbits are commonly used for production of antibodies because they are relatively inexpensive and easy to handle. Although the safety and efficacy of using CpG-ODN as an immunological adjuvant to boost antibody production in rabbits have been investigated with favorable results [25], the function and interaction of rabbit TLR9 (rabTLR9) with CpG-ODN has not yet been investigated. In this study, we cloned and characterized rabTLR9 cDNA, and comparatively investigated the activation of rabbit, mouse, and human TLR9 by CpG-ODN.

2. Materials and methods

2.1. Reagents and antibodies

CpG-ODN, 4′,6 diamidino-2-2 phenylindole (DAPI) and Alexa-594-conjugated anti-mouse antibody were purchased from Invitrogen (Carlsbad, CA). FITC-conjugated CpG-2006 was purchased from Invivogen (San Diego, CA). Chloroquine and anti-flag M2 monoclonal antibody were purchased from Sigma (St. Louis, MO). Rabbit total RNAs from different tissues were purchased from Zyagen Laboratory (San Diego, CA).

2.2. Rapid amplification of cDNA ends

A modified rapid amplification of cDNA ends (RACE) method was performed using GeneRacer kit as described in the manual provided by the manufacturer (Invitrogen, Carlsbad, CA) to determine the 5′end cDNA sequence of rabTLR9. Briefly, rabbit spleen total RNA was treated with calf intestinal phosphatase and tobacco acid pyrophosphatase. After the selective ligation of GeneRacer RNA oligonucleotide to the 5′-ends of decapped mRNA, first strand cDNA synthesis was carried out with Superscript III reverse transcriptase. cDNA fragment encoding the 5′-end sequence of rabTLR9 was PCR amplified from the GeneRacer-generated first-strand cDNA library with a GeneRace 5′-primer and a primer specific to *rabTLR9* gene (5′-GGCAGGAACTGCGAGCCGTTGAC-3′) identified from nucleotide sequence of genomic DNA encoding *rabTLR9* (accession number: AAGW02044169). The generated cDNA containing 5′-end of rabTLR9 was ligated into a T/A cloning vector (Invitrogen, Carlsbad, CA) for sequencing.

2.3. Cloning and expression constructs for rabTLR9

Full-length rabTLR9 cDNA was cloned by PCR amplification from a rabbit first-strand cDNA library. This library was prepared from rabbit spleen total RNA using a Superscript preamplification kit (Invitrogen, Carlsbad, CA). PCR amplifications were performed using an Expand HI Fid PCR kit (Roche, Indianapolis, IN). The generated DNA fragment containing rabTLR9 was ligated into a T/A cloning vector (Invitrogen, Carlsbad, CA) for sequencing. To construct expression vector for rabTLR9, cDNA fragment was generated by PCR amplification from rabbit first-strand cDNA library with forward and reverse primers designed based on the DNA sequence for the complete coding region (amino acid residue 1-1028) of rabTLR9. The forward primer contains a KpnI site and has the sequence 5′-GGAAGGG TACCGCCACCATGTGTCCCTGTCGAGGAGCCC-3′. The reverse primer contains a SpeI site and has the sequence 5′-GGAAGACTAGTTTCAGCTGTGGGCCC CCGGCAG-3′. This PCR product was subcloned into a PEF6 expression vector (Invitrogen, Carlsbad, CA) containing a nucleotide sequence for expression of a Flag tag fusing to the c-terminal of the expressed rabTLR9.

2.4. Bioinformatic analysis

DNA sequence analysis was performed with VectorNTI software (Invitrogen, Carlsbad, CA) [26] and the GCG Wisconsin Package (Accelrys, San Diego, CA) [27]. Phylogenetic analysis for TLR9 from multiple species was performed with ClustalW2 software (http://www.ebi.ac.uk/Tools/clustalw2/) [28].

2.5. RT-PCR analysis

RT-PCR analysis was performed as previously described [29]. The sequences of the gene-specific primers for rabTLR9 are 5′-GCAACCTCACCCACCTGTCACTC-3′, and 5′-CACC AGGTTGTTCCGAGACAGGTC-3′. Primers (5′-TGAAGGTCGGAGTCAACGGATTTGG TCG-3′, and 5′-CATGTGGGCCATGAGGTCCACCAC CAC-3′) based on human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed for analysis of rabGAPDH. PCR products were visualized by electrophoresis on a 1% agarose gel after staining with ethidium bromide.

2.6. Cell culture, transfection, and rabbit, mouse, and human TLR9 activation assays

Human embryonic kidney (HEK) 293 cells were maintained in DMEM supplemented with 10% fetal bovine serum. To perform rabbit, mouse, and human TLR9 activation assays, the cells were seeded on 12-well plates and co-transfected on the following day using PolyJet (SignaGen Laboratories, Rockville, MD) with TLR9 (rabTLR9, mTLR9, and hTLR9) expression vector (50 ng), β-galactosidase plasmid (50 ng), and an NF-κB driven luciferase reporter plasmid (50 ng), and supplemented with empty pBluescript vector to 500 ng. These cells were treated with 2 μM of various CpG-ODN or different concentrations of CpG-ODN as indicated on the next day for 6 h and lyzed. Luciferase activity in each sample was determined using reagents from Promega Corp. (Madison, WI). Relative luciferase activities were calculated as fold induction compared to an unstimulated control. The data are expressed as the mean ± SD ($n = 3$).

2.7. Immunofluorescence staining

For detection of rabTLR9 localization, HEK293 cells were transiently transfected with expression vector for flag-tagged rabTLR9 overnight. These cells were treated with and without 2 μM of FITC-conjugated CpG-2006 for 3 h and then fixed in 2% paraformaldehyde and permeabilized with 0.2% of Triton X-100 in PBS. After PBS washing, cells were stained with an anti-flag M2 monoclonal antibody, followed by Alexa-594-conjugated anti-mouse antibody (Invivogen, Carlsbad, CA). To visualize nuclei, cell were stained with 5 ng/ml DAPI (Invivogen, Carlsbad, CA). Samples were observed with an IX70 inverted immunofluorescence microscope (40×) (Olympus America, Melville, NY).

3. Results

3.1. Molecular cloning of rabTLR9

Genomic DNA encoding *rabTLR9* (accession number: AAGW02044169) was identified in a search of NCBI databases with a Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This sequence was aligned with the nucleotide sequence of mTLR9 to determine the 3′-end nucleotide sequence of rabTLR9. A rapid amplification of cDNA ends (RACE) approach with primers based on the genomic DNA sequence was adopted to determine the 5′-end cDNA sequence of rabTLR9. Based on these sequences, 5′-end and 3′-end primers were designed for PCR amplification of rabTLR9 cDNA from a rabbit spleen first strand cDNA library. A full-length rabTLR9 cDNA was cloned. The cDNA sequence was submitted to the GenBank database under the accession number HM448400.

3.2. Sequence analysis of rabTLR9

This rabTLR9 cDNA contains 3298 base pairs (bps). The start codon (ATG) is located at 78–80 bp and that stop codon (TAG) at 3162–3164 bp. The predicted open reading frame from 78 bp to 3164 bp encoding 1028 amino acid residues (Fig. 1). This protein sequence was aligned with hTLR9 protein sequence. The identity between these two proteins was 75%. hTLR9 consists of an extracellular domain (ECD), a transmembrane domain, and a Toll/IL-1 (TIR) cytosolic domain. The extracellular domain comprises an undefined region, 25 copies of leucine repeats (LRRs), and a C-terminal leucine-rich repeat (CT-LRR) [30]. These architectural features are present in rabTLR9 (Fig. 1). Thus, these suggest that TLR9 is highly conserved in these two species.

3.3. Phylogenetic analysis of TLR9 from different species

To further compare TLR9s from different species, we carried out a phylogenetic analysis. Protein sequences of human, mouse, rat, rabbit, horse, dog, cat, procine, bovine, and sheep TLR9 were aligned with a ClustalW2 computer program, and an evolution tree was drawn (Fig. 2). Rabbit TLR9 is in a rodent clade containing mouse, rat, and rabbit TLR9s. However, although these three TLR9s are more closely related to each other in this phylogenetic analysis, the overall protein identities of rabTLR9 to mTLR9 (70%) and to rat TLR9 (68%) were lower than that to hTLR9 (75%), as well as lower than to those in the domestic animal clade containing TLR9s from horse (77%), dog (77%), cat (77%), procine (75%), bovine (73%), and sheep (73%) (Fig. 2).

3.4. Tissue distribution analysis

Expression of rabTLR9 in different tissues was analyzed by RT-PCR with gene-specific primers. Transcript of this TLR was barely detected in brain, heart, kidney, colon, stomach, uterus, and testicle. In contrast, this TLR was expressed in lung, liver, thymus, spleen, pancreas, and ileum (Fig. 3). These results suggest that, similar to the expression profile of hTLR9 in human tissues [31], rabTLR9

Fig. 1. Architecture and amino acid sequence of rabTLR9. (A) Predicted architecture of rabTLR9. LRRs, leucine-rich repeats; UD, undefined region; LRR-CT, C-terminus LRR; TM, transmembrane domain; TIR, Toll/IL-1R cytoplasmic domain. The scale shows amino acid residue numbers. (B) Deduced amino acid sequence of rabTLR9. The LRRs are underlined. UD is dotted underline. LRR-CT is in bold type and underlined. TM is double underlined. TIR is double dotted underline.

is preferentially expressed in immune cells rich tissues (Fig. 3).

3.5. Cellular localization of rabTLR9

Human and mouse TLR9s were shown to be expressed in intracellular vesicles, including endoplasmic reticulum, endosomes, and lysosomes [7,8]. To investigate cellular localization of the rabTLR9 and to determine its interaction with CpG-ODN, 293 cells were expressed with this TLR, treated with/without FITC-conjugated CpG-2006, and visualized by confocal microscopy following immunofluorescence staining. The result showed an intracellular localization of rabTLR9 and co-localization of CpG-2006 with this TLR in intracellular vesicles (Fig. 4). The intracellular activation of rabTLR9 by CpG-ODN was further investigated with a TLR9 activation assay. In this assay 293 cells were co-transfected with a rabTLR9 expression vector and a luciferase reporter gene driven by NF-κB activity. These cells were treated with different concentrations of chloroquine and then stimulated with CpG-2006. Chloroquine which has been shown to inhibit hTLR9 activation, is an agent that prevents endosomal acidification and thus prevents endosome maturation [16,32]. The result indicated that chloroquine effectively blocked CpG-ODN-induced rabTLR9 activation at 1 μM concentration. This further confirmed the localization and activation of rabTLR9 inside the cells.

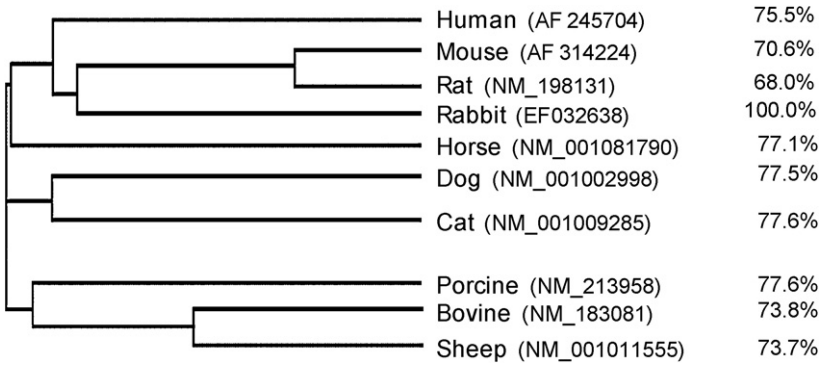

Fig. 2. Phylogenetic tree of the TLR9 genes. The phylogenetic tree was derived from an alignment of protein sequence of the TLR9 members by using a ClustalW2 program. GenBank accession number of each TLR9 protein sequence used in this phylogenetic analysis is included in parentheses. Percentages indicate the TLR9 protein sequence identities between rabbit and other species.

3.6. Activation of rabTLR9, mTLR9, and hTLR9 by CpG-ODN

We turned to investigate the activation of rabTLR9 by different CpG-ODN with the TLR9 activation assay, and compared the activation profiles of rabTLR9 with those of hTLR9 and mTLR9. As listed in Table 1, CpG-2006 and CpG-2007 contain three copies of GTCGTT motifs, with different spacing between the motifs. CpG-2429 and CpG-2395 contain a copy of the GTCGTT motif but slightly differ in their 3′ palindromes. CpG-1826, CpG-2000, and CpG-2002 all contain two copies of GACGTT motifs with different spacing.

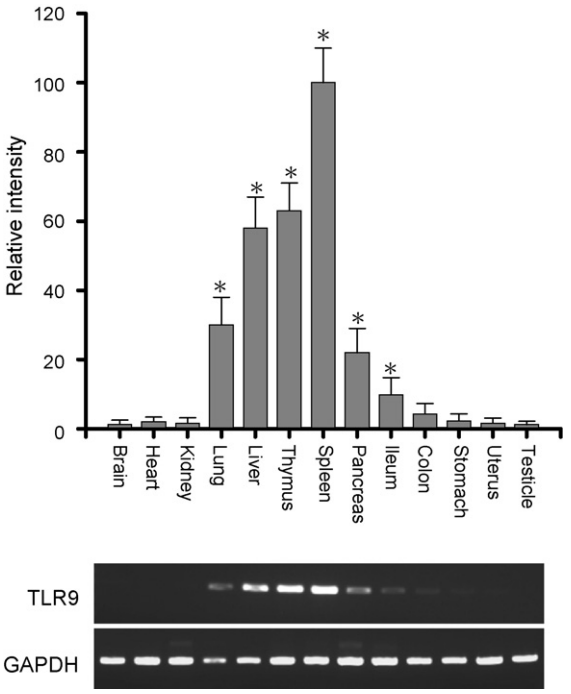

Fig. 3. Expression of rabTLR9 in rabbit tissues. Expression of the indicated genes was analyzed by RT-PCR. Data presented are relative intensity of PCR product for rabTLR9 normalized to GAPDH, and presented as mean ± SD ($n = 3$). *$P < 0.05$ *vs.* the expression in brain. Blots in the figure shows a representative RT-PCR.

Previous studies have shown that GTCGTT is an optimized motif for activation of hTLR9 and that CpG-ODN containing the GACGTT motif preferentially activates mTLR9, although the activity of CpG-ODN is also dependent on the spacing, position, and surrounding bases of these motifs. [9–13]. As expected, hTLR9 and mTLR9 displayed distinct responding profiles to these GTCGTT and GACGTT motifs containing CpG-ODN. hTLR9 was activated by CpG-2006 and CpG-2007 but not by CpG-1826, CpG-2000, and CpG-2002. In contrast, CpG-1826, CpG-2000, and CpG-2002 were more potent in activation of mTLR9 compared to other CpG-ODN (Fig. 5). Distinct from hTLR9 and mTLR9, rabTLR9 responded almost equally well in these experiments to the GTCGTT motifs containing CpG-ODN (CpG-2006 and CpG-2007) and to the GACGTT motifs containing CpG-ODN (CpG-1826, CpG-2000, and CpG-2002) (Fig. 5).

To further confirm this result, dose responses of rabTLR9 to the CpG-2006, CpG-2007, CpG-1826, and CpG-2000 stimulations were investigated. The results showed a very similar dose–response curve of rabTLR9 activations by these CpG-ODN (Fig. 6). This suggests rabTLR9 indeed did not discriminate these CpG-ODN and that the TLR9s from rabbit, mouse, and human have a different CpG-ODN recognition profile.

3.7. Effective concentrations of CpG-ODN in activation of rabTLR9, mTLR9, and hTLR9

Although rabTLR9 was activated by those CpG-ODN optimized for activation of hTLR9 and mTLR9, the activations were not as strong as the activations of hTLR9 and mTLR9 by their optimized CpG-ODN (Fig. 5). CpG-2007 has been shown to induce a more safer and potent antibody response than other adjuvants in rabbits [25]. We investigated the activations of rabTLR9 under different concentrations of CpG-2007 and compared the results with the activations of mTLR9 and hTLR9 by different concentrations of CpG-1826 and CpG-2006. The EC_{50} for CpG-1826 to activate mTLR9 was 0.15 μM, and the EC_{50} for CpG-2006 to activate hTLR9 was 0.75 μM, whereas it was 1.50 μM for CpG-2007 to activate rabTLR9 (Fig. 7). These findings, in combination with the results presented in Fig. 6, suggest a

Table 1
The nucleotide sequences of the CpGODNs used in the present study.

CpG-ODN	sequence 5'- 3'	
CpG-685	tcgtcgacgtcgttcgttctc	
CpG-2006	tcgtcgttttgtcgttttgtcgtt	gtcgtt motif containing CpG-ODN
CpG-2007	tcgtcgttgtcgttttgtcgtt	
CpG-2429	tcgtcgttttcggcggccgccg	
CpG-2395	tcgtcgttttcggcgcgcgccg	
CpG-2007-GC	tgctgcttgtgcttttgtgctt	
CpG-2002	tccacgacgttttcgacgtt	gacgtt motif containing CpG-ODN
CpG-2000	tccatgacgttcctgcagttcctgacgtt	
CpG-1826	tccatgacgttcctgacgtt	
CpG-1826-GC	tccatgagcttcctgagctt	

higher concentration is required for CpG-ODN to effectively activate rabTLR9.

4. Discussion

The successful cloning and characterization of rabTLR9 cDNA in the present study revealed that rabTLR9 shares a high homology in its amino acid sequence with those TLR9 from other mammals. In addition, all the architectural characteristics of a TLR9, including the undefined region in the ECD domain, are conserved in this rabTLR9. In general, the structural features of a TLR include a cytosolic TIR domain, a transmembrane domain, and an ECD containing multiple LRRs. Each LRR consists of a β-sheet followed by an α-helix. These LRRs are arranged to form a horseshoe-shaped solenoid structure with the α-helixes on its outer convex surface and the parallel β-sheets on its concave surface, providing a ligand-binding site. A unique feature for the members of the TLR7, TLR8, and TLR9 subfamily is an insertion of the undefined region in between LRR14 and LRR15. This region contains about 40 amino acid residues and is the only one in these TLRs that shows a high degree of variation in each TLR from different species [30,33,34]. The hyporesponses of two rodent TLR8s from mouse and rat to their ligands has been attributed to the lack of a five amino acid residues motif in this region, presumably because this region plays a role as a hinge for building a three-dimensional conformation for a high-affinity binding to ligands [35]. The conserved undefined region in rabTLR9 implicated a structural integrity of this TLR.

While other TLRs are expressed on the cell surface, TLR3, TLR7, TLR8, and TLR9 are expressed in intracellular vesicles [7,8]. A tyrosine-based vesicle-targeting motif, YNEL in the TIR domain was identified to direct vesicular localization of hTLR9 [36]. This motif is conserved in rabTLR9 from the amino acid residue 884–887. Our results suggest that the rabTLR9 acts similarly to TLR9s from other species: as an endolysosomal sensor in responding to CpG-ODN activations. This notion was supported by the intracellular localization of rabTLR9 and by the co-localization of CpG-ODN with this TLR in intracellular vesicular compartments. In addition, treatment of cells with chloroquine rendered rabTLR9 hyporesponse to GpG-ODN stimulations. Chloroquine is an endosomal maturation inhibitor. This result suggests that endosomal maturation is required for the interaction of CpG-ODN with rabTLR9.

Despite the conservation of all structural features and the cellular localization being identical to that of the other TLR9s, the rabTLR9 displayed a CpG-ODN-responding profile distinct from those of hTLR9 and mTLR9. Human TLR9 responds to CpG-ODN containing GTCGTT motif, such as the CpG-2006 and -2007, but had no response to those containing GACGTT motif, such as CpG-1826, -2000, and -2002. In contrast, mTLR9 was more responsive to those

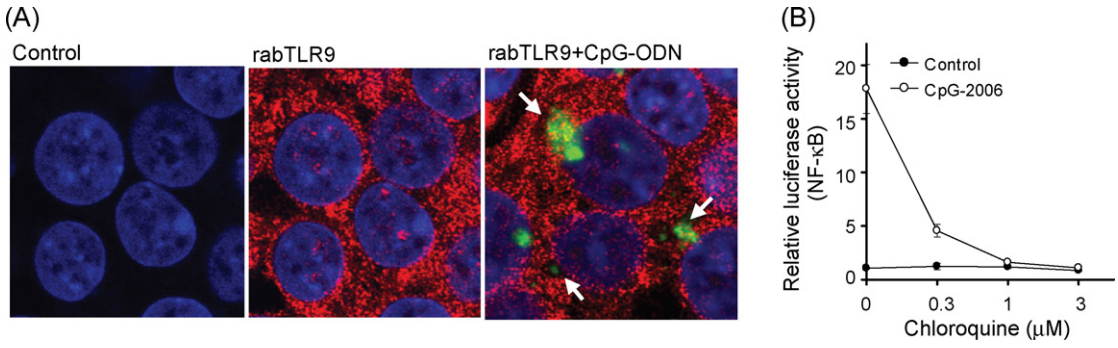

Fig. 4. Intracellular localization and activation of rabTLR9. (A) 293 cells were transfected with/without expression vector for rabTLR9 and treated with/without FITC-conjugated CpG-2006. Cellular localization of rabTLR9 was visualized by immunofluorescence staining using anti-flag M2 mAb, followed by an Alexa 594-labeled anti-mouse antibody (red). Nuclei were stained by DAPI (blue). Arrows show larger CpG-ODN vesicles, indicating colocalization with rabTLR9. (B) 293 cells were co-transfected with expression vector for rabTLR9 and NF-κB luciferase-reporter gene, and treated with CpG-2006 under different concentrations of chloroquine. Relative luciferase activities were determined. Data shown represent mean ± SD ($n = 3$).

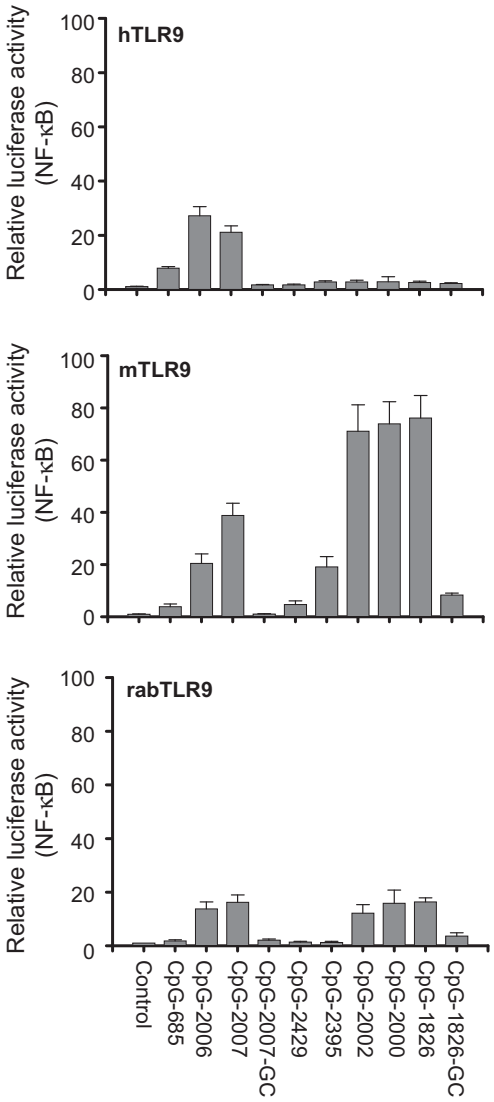

Fig. 5. Activation of human, mouse, and rabbit TLR9 by different CpG-ODN. 293 cells were transfected with expression vector for TLR9 from different species as indicated plus an NF-κB luciferase-reporter gene, and treated with 2 μM of CpG-ODN. Relative luciferase activities were determined. Data shown represent mean ± SD ($n = 3$).

GACGTT motifs containing CpG-ODN. Distinct from hTLR9 and mTLR9, the rabTLR9 displayed a broader ligand recognition profile. Rabbit TLR9 is activated by those CpG-ODN containing either GTCGTT motif or GACGTT motif. The CpG-ODN-responding profiles of these TLR9s from different species in the cell-based assay were consistent with previously observed immunostimulatory effects of these CpG-ODN on peripheral blood mononuclear cells (PBMCs) and splenocytes isolated from the corresponding species. In their study, Rankin et al. showed that GACGTT is an optimized motif for activation of mouse cells, and that GTCGTT is an optimized motif for activation of human cells and cells from a variety of domestic animals, including sheep, goat, horse, pig, and dog, whereas no preference for these two motifs was seen for rabbit cells [37].

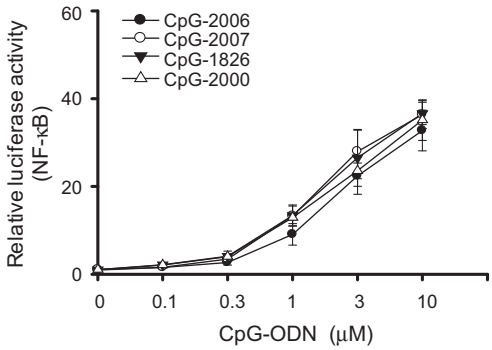

Fig. 6. Concentration dependent activation of rabTLR9 by different CpG-ODN. 293 cells were co-transfected with expression vector for rabTLR9 and an NF-κB luciferase-reporter gene, and treated with various concentrations of CpG-ODN. Relative luciferase activities were determined. Data shown represent mean ± SD ($n = 3$).

Species-specific ligand recognitions are frequently seen for TLRs from different species. For example, the hTLR2/1 and hTLR2/6 receptor complexes are activated by triacyl-lipopeptides and diacyl-lipopeptides, respectively, in human cells, whereas both lipopeptides activate a bovine TLR2/1 complex. *Rhodobacter sphaeoides* activates TLR4 signaling in hamster and horse, but acts as an antagonist in mouse and human. Chicken TLR5 generates stronger responses to flagellin from *Salmonella enterica* serovar Typhimurium than hTLR5. Activation of hTLR8 by R848 elicits potent immune responses, while mTLR8 is not responsive to ligand stimulation in the absence of polyT-ODN [34,35]. In the present study we show that the mouse, human, and rabbit TLR9 display different ligand recognition profile and have a different kinetic in responding to CpG-ODN stimulation. This provides another example of the species-specific ligand recognition property of TLRs.

The molecular basis underlying these species-specific ligand recognitions of TLR9 is still poorly understood. Soluble cofactors such as high-mobility group box (HMGB) and granulin have been shown to bind DNA and to be

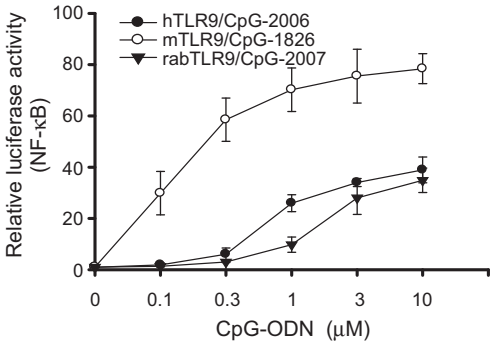

Fig. 7. Effective concentration for activation of human, mouse, and rabbit TLR9 by their optimized CpG-ODN. 293 cells were co-transfected with expression vector for human, mouse, or rabbit TLR9 and an NF-κB luciferase-reporter gene. The cells were treated with various concentrations of the indicated CpG-ODN. Relative luciferase activities were determined. Data shown represent mean ± SD ($n = 3$).

essential for TLR9 activation [38,39]. Whether these co-factors play a role to mediate the specific-specific ligand recognition is unclear. Nevertheless, in this study the cell-based TLR9 activation assay that was established by transfection of TLR9 from different species into 293 cells copied their species-specific ligand recognition property, suggesting that the intrinsic structural difference of TLR9 may contribute to the species-specific response to CpG-ODN. Structure–function analysis by mutagenesis and homology modeling suggest two binding regions on hTLR9 for CpG-ODN. One is near the N-terminus, involving the LRR insertions of LRR2, 5, and 8, as well as a negatively charged patch around K51 and R74; the other is centrally located around D535 and Y537 [40]. Nevertheless, this model only suggested the binding sites for CpG-ODN, but did not provide any structural basis for the specific-specific ligand binding. Previous studies with TLR8 from multiple species suggest that the ligand binding and the species different ligand binding may be determined by multiple layers of molecular determinants of a TLR [35]. Similar to this, the different ligand recognition profile among the mouse, human, and rabbit TLR9 discovered in present study might also be determined by molecular determinants different from that required for building the CpG-ODN binding sites in TLR9. These molecular determinants have yet to be explored.

Rabbits are commonly used for production of polyclonal antibodies. Moreover, a technique for generation of rabbit monoclonal antibodies has been developed and has been investigated as a means for generating therapeutic antibodies [41,42]. Freund's complete adjuvant is widely utilized for effectively boosting antibody production in rabbits. Nevertheless, because this adjuvant causes inflammatory responses and lesions around the injection sites it is allowed only for the first immunization, and incomplete adjuvant is used for the boost immunizations. Therefore, efforts have continued to find more efficient adjuvants for maximizing antibody response and minimizing adverse effects [43–45]. CpG-ODN are potent immune stimuli. In combination with other adjuvants or carriers, CpG-2007 have been shown to induce stronger antibody response and less toxicity than the Freund's adjuvant in rabbits [25]. In the present study, we found that CpG-1826, CpG-2000, and CpG-2002 optimized for mouse TLR9, and CpG-2006 and CpG-2007 optimized for human TLR9 are nearly equally strong in activation of rabTLR9, which is consistent with previous study's findings on the *in vivo* immune stimulatory activities of these CpG-ODN [37] and suggests that these CpG-ODN might have equivalent potency in generating antibody responses in rabbits. Nevertheless, our results also showed the requirement of a higher EC_{50} of the CpG-2007 to activate rabTLR9 than the EC_{50} of the CpG-1826 to activate mTLR9, and the EC_{50} of the CpG-2006 to activate hTLR9. In general, the activity of CpG-ODN is determined by its sequence context—including its length, the number of CpG motifs, and the spacing, position, and surrounding bases of these motifs [9–13]. Whether these CpG-ODN can be further optimized to reach higher activity in generating antibody response in rabbits would require investigation. For this the rabTLR9 activation assay would be a useful tool.

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