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Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9

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Abstract

Synthetic oligodeoxynucleotides (ODN) expressing non-methylated "CpG motifs" patterned after those present in bacterial DNA have characteristic immunomodulatory effects. CpG DNA is recognized as a pathogen-associated molecular pattern, and triggers a rapid innate immune response. CpG ODN are being harnessed for a variety of therapeutic uses, including as immune adjuvants, for cancer therapy, as anti-allergens, and as immunoprotective agents. The signal transduction pathway mediated by the engagement of CpG DNA with Toll-like receptor 9 (TLR9) is shared with other members of the TLR family. Recent studies demonstrate that formation and maturation of CpG DNA-containing endosomes are regulated by phosphatidylinositol 3 kinases and the Ras-associated GTP-binding protein, Rab5, which are essential for the initiation of TLR9-mediated signaling.

Keywords: CpG DNA; TLR 9; PAMP; Signal transduction

1. TLR9 is an essential element in CpG DNA-mediated cell activation

The discovery and characterization of the Toll-like receptor (TLR) family facilitated our understanding of the innate immune system. Accumulating evidence demonstrates that individual TLRs identify determinants known as pathogen-associated molecular patterns (PAMPs) that are expressed at high frequency by infectious microorganisms but rarely (if at all) by host cells [1]. For example, lipopolysaccaride (LPS) present in the surface membrane of Gram-negative bacteria engages the TLR4/MD-2 complex, while peptidoglycan (PGN) and bacterial lipoproteins (BLPs) present in the cell wall of Gram-positive bacteria engage TLR2 [2]. The interaction of a TLR with its PAMP ligand mediates an intracellular signal that results in the production of versatile chemical mediators and cell surface molecules directing innate as well as acquired immune responses [1,3,4].

All functionally characterized TLRs signal through a common pathway involving myeloid differentiation marker 88 (MyD88), IL-1R-associated kinase (IRAK), TNFR-associated factor 6 (TRAF6), TGFb-activated kinase1 (TAK1), and the kinases of IkB (IKK), IkB, and NF-kB [5,6]. Thus, TLRs are regarded as pattern recognition receptors (PRR) that sense PAMPs [1]. A series of studies support the conclusion that sequence motifs present at high frequency in bacterial but not mammalian DNA also act as PAMPs. Bacterial DNA triggers cells of the innate immune system to proliferate and become functionally active [7]. This immune stimulation is mediated by unmethylated CpG motifs present at high frequency in bacterial DNA, but rare in mammalian DNA [8,9]. The likelihood that CpG motifs act as PAMPs is further supported by evidence that the immune response they induce closely resembles that stimulated by LPS and PGN, and that responsiveness to CpG DNA is completely lost in MyD88 knock out (KO) mice [10]. Definitive evidence that CpG recognition is mediated by TLR9 was provided by studies involving TLR9 KO mice [11].

Our lab demonstrated that CpG recognition in humans is also mediated by TLR9 [12]. When several TLR9 null cells (HEK293, U87MG, HepG2, and CHOK1) were transfected with an hTLR9 expression plasmid (pCI-TLR9A) driving an NF-kB-dependent luciferase reporter plasmid, CpG DNA exposure resulted in a significant increase in luciferase activity. This stimulation was abrogated if the critical CpG motif was disrupted by inversion or methylation (see Table 1). When transfected with pCI-TLR9A, HEK293 cells produced IL-8 mRNA in response to CpG ODN but not to control

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Table 1
CpG ODN activate NF-kB expression in HEK293 cells transfected with hTLR9

Treatment	ODN sequence	Stimulation index
No Rx		1.0 + 0.2
2006	TCGTCGTTTTGTCGTTTTGTCGTT	8.0 + 0.4
K3	ATCGACTCTCGAGCGTTCTC	13.2 + 0.9
K3-flip	ATGCACTCTGCAGGCTTCTC	1.3 + 0.2
K3-methyl	AT^{m}CGACTCT^{m}CGAG^{m}CGTTCTC	1.7 + 0.1
1555	GCTAGACGTTAGCGT	2.3 + 0.2
1612	GCTAGATGTTAGCGT	2.0 + 0.2
1466	TCAACGTTGA	1.1 + 0.1
LPS		0.9 + 0.1
PGN		1.3 + 0.2

HEK293 cells were co-transfected with an hTLR9 expression vector plus an NF-kB-dependent luciferase reporter vector. After 24 h incubation with 1 μM of ODN or 1 μg/ml of LPS or PGN, luciferase activity in the cells was measured. The stimulation index reflects relative luciferase activity in cells treated with each agent vs. untreated cells. Note that ODNs 2006 and K3 contain sequences optimized for recognition by hTLR9, that K3-flip and K3-methyl represent controls in which critical CpG dinucleotides were inverted or methylated; and that ODNs 1555 and 1466 contain CpG motifs optimized for recognition by murine TLR9. Critical CpG dinucleotides are underlined. ^{m}C indicates a methyl cytosine.

ODN nor LPS, suggesting that TLR9 is an essential element in CpG DNA-mediated cellular activation [12]. Among microbial products and related compounds, CpG DNA is the only ligand for TLR9.

2. Association between CpG DNA and TLR9

The subcellular localization of CpG DNA with TLR9 was examined. HEK293 cells were transfected with a TLR9 construct tagged with a hemaggulutinin epitope (HA-TLR9). These cells were incubated with synthetic CpG ODN labeled with Cy3 plus FITC-labeled anti-HA antibody. CpG ODN and TLR9 molecules were visualized as red and green fluorescence, respectively, and their subcellular localization was assessed by confocal microscopy. In the absence of CpG DNA stimulation, TLR9 molecules localized to endocytic vesicles lying just below the plasma membrane [13]. Cy3-labeled CpG ODN associated with these vesicles, and also entered the nucleus within 10 min. By 2 h after the addition of CpG ODN to TLR9 expressing cells, the size and number of CpG ODN-containing endosomes increased significantly. Moreover, these vesicles relocated from near the plasma membrane to intracellular regions. In some cases, both CpG ODN and TLR9 molecules co-localized within the same endosomes (see Fig. 1) [12]. This distribution was not observed in cells transfected with a mutant form of HA-TLR9 lacking the intracellular domain (ICD) of TLR9, suggesting that the cryptic sorting signal present in the ICD and adequate conformation of TLR9 in the presence of the ICD are required for trafficking of TLR9 to CpG ODN-containing endosomes [12].

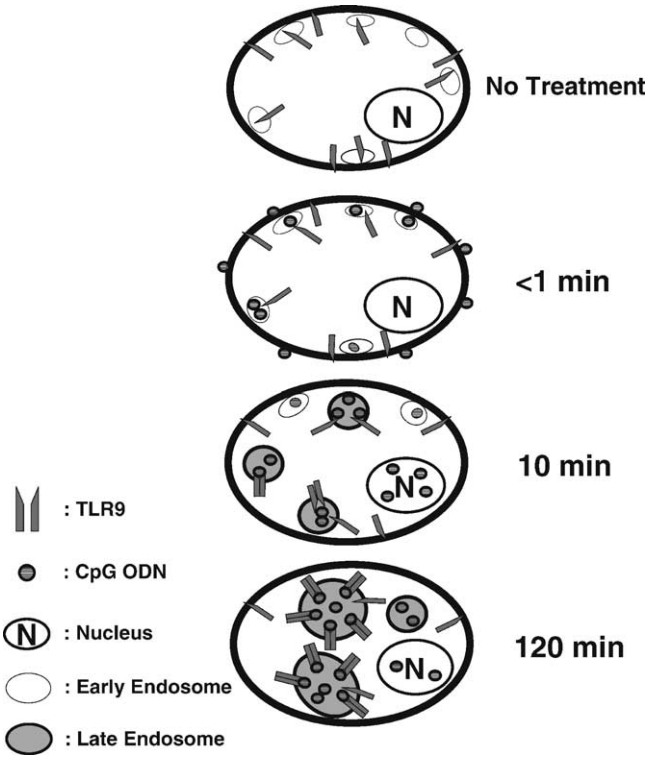

Fig. 1. Scheme of subcellular localization of Cy3-labeled CpG ODN and HA-TLR9. Under physiologic conditions, TLR9 molecules are located below the plasma membrane, presumably in vesicles. Cy3-labeled CpG ODN associate with the cell surface and are transported to these vesicles within 10 min. By 2 h the size and number of CpG ODN-containing endosomes increases significanty, and they relocate from just below the plasma membrane to intracellular regions. In some cases, the CpG ODN and TLR9 molecules co-localize within the same vesicles, recruit MyD88, and initiate the signal transduction process.

Investigators have examined the difference between TLR4- and TLR9-mediated signaling events. Results from studies involving GFP-tagged MyD88 (MyD88-GFP) in RAW264.7 macrophages indicate that the TLR4-mediated signal initiates near the plasma membrane, while the TLR9-mediated signal initiates around CpG DNA-containing endosomes [14]. These studies showed that MyD88-GFP was recruited into the lysosomal-associated membrane protein 1 (Lamp-1)-positive endosomal compartment >45 min after CpG ODN treatment.

Immune cell activation by CpG DNA and co-localization of CpG DNA with MyD88-GFP were prevented when the early steps of endocytosis were inhibited by treatment with monodansylcadaverine. The same inhibition was elicited by a dominant negative mutant of the Ras-associated GTP-binding protein, Rab5, that blocks homotypic fusion of the early endosome and endosomal trafficking [14]. These findings suggest that both formation and maturation of CpG ODN-containing endosomes are critical for the initiation of TLR9-mediated signaling.

Over evolutionary periods, TLR9 molecules expressed by different species have diverged. This has led to differ-

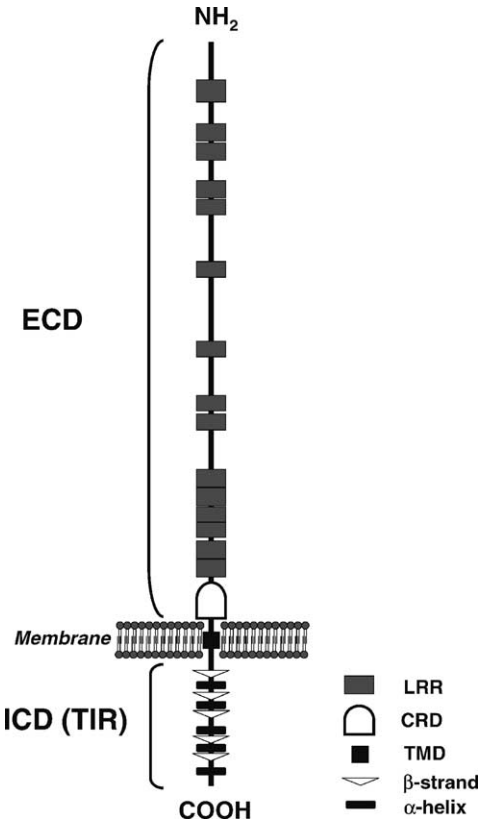

Fig. 2. Schematic representation of the molecular structure of TLR9. TLR9 is a type I membrane protein which consists of an extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular domain (ICD). Leucine-rich repeats (LRRs) and a cysteine-rich domain (CRD) are present in the ECD, whereas the Toll/IL-1R homologous region (TIR) exists in the ICD.

ences in the precise sequence motif (CpG dinucleotide plus flanking regions) that optimally stimulate the innate immune system of different animals. For example, HEK293 cells transfected with murine TLR9 are more responsive to CpG motifs optimized for activity in mice (GACGTT) than humans (GTCGTT). Similarly, cells expressing human TLR9 responded optimally to GTCGTT rather than GACGTT. These findings confirm the importance of TLR9 as a receptor for CpG DNA, and suggest that this receptor accounts for species-specific differences in the response to bacterial DNA [15].

3. Cellular signaling mediated by TLR9

Computer-based analysis indicates that TLR9 is a type I membrane protein which shares common structural characteristics with other members of the TLR family. Fifteen leucine-rich repeats and one cysteine-rich domain exist within the extracellular domain (ECD) of TLR9, and a Toll/IL-1R homologous region (TIR) exists within the ICD (see Fig. 2). Studies using truncated forms of hTLR9 indicate that 32 amino acids at the COOH-terminus form a fifth a-helical structure that is critical to signaling. Co-transfection of HEK 293 cells with wild type TLR9 plus ICD deletion mutants suggest that TLR9 may function after oligomerization through the ECD interaction [12].

TLR9-mediated signaling proceeds through MyD88, an adaptor protein recruited to the TIR, which then activates the IRAK1-TRAF6-TAK1 pathway [5,6,12]. Unlike TLR4-mediated signaling, the TIR domain-containing adaptor protein/MyD88-adapter-like (TIRAP/MAL) is not involved in TLR9-mediated signaling [16,17]. Recently, a novel adapter molecule associated with MyD88-independent as well as MyD88-dependent pathways was identified [18]. Ongoing studies suggest that this molecule, TIR domain-containing adapter inducing IFN-b (TRIF), is also involved in TLR9-mediated signaling (unpublished observation).

The TLR9 signaling cascade involves mitogen-activated protein kinases (MAPKs), such as p38, c-Jun NH_2-terminal kinase (JNK), extracellular receptor kinase (ERK), and NF-kB-inducing kinase (NIK)-IKK-IkB pathways [19–21]. The activation of ERK by CpG DNA contributes to the production of IL-10 by macrophages, but is not active in dendritic cells (DCs) or B cells [20,22]. The signaling cascade culminates in the activation of several transcription factors including NF-kB, activating protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and cAMP-responsive element-binding protein (CREB), which directly upregulate cytokine/chemokine gene expression (see Fig. 3) [20,23–25]. In macrophages, CpG DNA also induces IFN-b production, which then upregulates STAT1 phosphorylation and IP-10 production through IFN-a/b receptor in an autocrine manner [26].

Studies using chloroquine (CQ) or wortmannin (WM) showed that these agents could block CpG DNA/TLR9 signaling but not LPS/TLR4 signaling [23,27,28]. Since cell surface binding and uptake of an ODN is not influenced by the presence of a CpG motif, endosomal maturation, which is the target of CQ, is believed to be an essential step in signaling [23,28]. Taken together with the data on the subcellular distribution of CpG DNA described above, co-localization of CpG DNA with TLR9 in endosomal vesicles, and the accompanying maturation and movement of those vesicles, seems to be involved in signaling initiation.

Although one group reported that the suppression of CpG DNA signaling by WM reflected the inhibition of DNA-dependent protein kinase (DNA-PK) [29], others find that DNA-PK KO mice and SCID mice respond normally to CpG DNA [27,30]. We observed that WM treatment led to a reduction in the size and number of endosomes containing both TLR9 and CpG ODN, suggesting that phosphatidylinositol 3 kinases (PI3K), which are also targets of WM, are involved in vesicular trafficking of CpG DNA [27]. Indeed, Rab5-mediated recruitment of class III PI3K (PI3K (III)) leads to the production of PI(3)P in the endosomal membrane, which binds to the FYVE domain of early endosome antigen 1 (EEA1), recruiting it on to the membrane. The recruited EEA1 also associates

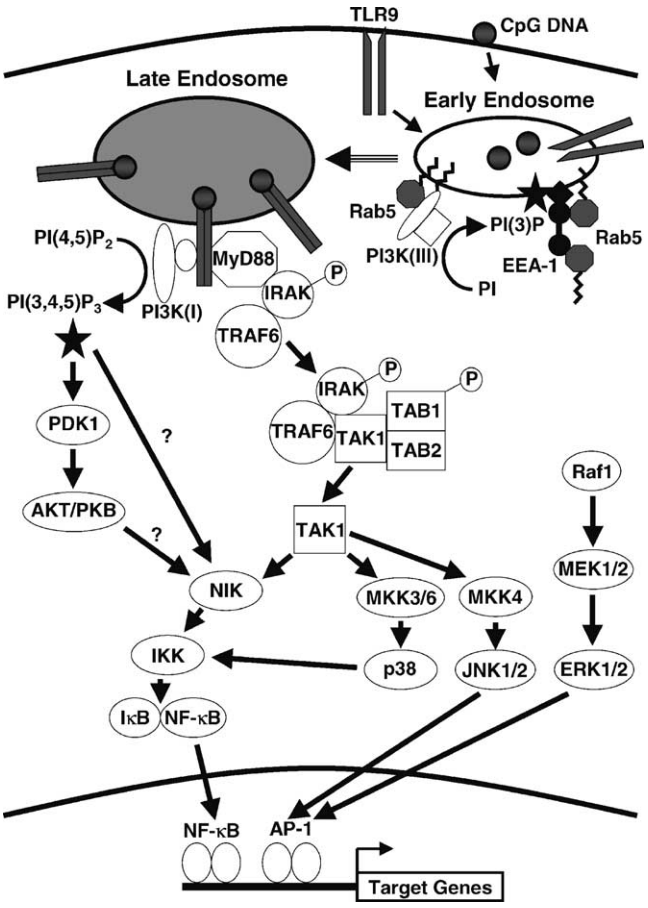

Fig. 3. Scheme of CpG DNA/TLR9-mediated cellular signaling. Class III PI3K (PI3K (III)), EEA1, and Rab5 mediate the trafficking and maturation of endosomes containing CpG DNA and TLR9, by which TLR9 transduces intracytoplasmic signal. The signal initiates with the recruitment of MyD88 to the TIR, which then activates IRAK-TRAF6-TAK1 complex. This leads to the activation of both MAPKs (JNK1/2 and P38) and IKK complex, culminating upregulation of transcription factors including NF-kB and AP-1. Raf1-MEK1/2-ERK1/2-AP-1 pathway is involved in CpG DNA-induced IL-10 production inmacrophages. The alternative pathway mediated by class I PI3K (PI3K (I))-PDK1-AKT/PKB is also suggested to be involved in TLR9-mediated cellular activation.

with Rab5 and regulates homotypic fusion and trafficking of early endosomes (see Fig. 3) [31–33]. The PI(3, 4, 5)P3, product of class I PI3K (PI3K (I)), has been demonstrated to activate a signaling cascade consisting of 3′-phosphoinositide-dependent kinase-1 (PDK1) and the protein kinase Akt/protein kinase B (AKT/PKB) [34,35]. Ligand-induced association of TLR2 ICD and PI3K (I) was reported to activate the AKT/PKB-NF-kB pathway [36]. CpG DNA also induces phosphorylation of AKT/PKB thereby inhibiting apoptosis in DCs, an effect that is reversed by a PI3K inhibitor, LY294002 [37]. However, recent data demonstrate that DN-p85a, which specifically blocks the function of PI3K (I), but neither DN-PDK1 nor DN-AKT/PKB, inhibits TLR9-mediated NF-kB activation in HEK293 cells (unpublished observation). This suggests that (1) PI3K (I) also regulate vesicular trafficking of CpG DNA and TLR9 and/or (2) another pathway mediated by PI3K(I) but not through the PDK1-AKT/PKB pathway is involved in TLR9-mediated NF-kB activation in HEK293. PI3Ks and their second messengers therefore seem to play pivotal roles at distinct steps (i.e. vesicular trafficking for the association between CpG DNA and TLR9 and the signaling pathway directing AKT/PKB activation) in CpG DNA/TLR9-mediated cellular activation.

4. Expression of TLR9 mRNA

The h*TLR9* gene consists of 2 exons, and there are two distinct spliced forms of hTLR9 mRNA. The biexonic protein, designated TLR9A, has 57 additional amino acids including a signal peptide when compared to the monoexonic protein (TLR9B) [38]. TLR9A mediates CpG DNA signaling, whereas TLR9B does not [12].

Northern blot analysis of various tissues including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis indicated that murine TLR9 (mTLR9) transcripts were most abundantly expressed in spleen [11]. Among cell types, hTLR9 mRNA is highly expressed in plasmacytoid precursor DCs ($CD11c^{-}$, $CD123^{+}$) and B cells, and to a lesser extent in monocytes [39–41]. Upon IFNg treatment, both TLR9 mRNA expression and responsiveness to CpG DNA are up-regulated in PBMC [12]. LPS also up-regulates mTLR9 mRNA expression in RAW264.7 macrophages, while colony stimulating factor-1 down-regulates constitutive mRNA expression in bone marrow-derived macrophages [42,43]. Sequence analysis of the h*TLR9* gene promoter region indicates that there are putative *cis*-elements including a TATA box, GC box, and consensus motifs for C/EBP. Transcription factors selectively expressed in a B cell linage and/or those upregulated by IFN-g or LPS may be involved in the expression of h*TLR9* gene.

5. Concluding remarks

Immune cells recognize CpG motifs using a member of the same family of TLR receptors that interact with other PAMPs, including PGN, glycolipid, and lipoprotein. Although various members of the TLR family share common structures, recent data suggest that distinct receptor–ligand associations and signaling pathways may be utilized by each member of this family. Several questions concerning the mechanism underlying CpG DNA/TLR9-mediated cellular activation currently persist. First, how do PI3Ks and their second messengers regulate trafficking and maturation of endosomes containing CpG DNA and TLR9 for signal initiation? Second, what is the precise mechanism responsible for the physical association between CpG motifs and TLR9? There is evidence that a structural change involving the TLR9 ECD and CpG motifs occurs in the acidified and Ca^{++} rich environment of the mature endosome that facil-

itates their association. In addition, various cofactor(s) may interact with the CpG motif and regulate this association. Third, a distinct class of CpG DNA such as "D"-type ODN (also known as CpG-A ODN) induces only modest NF-kB activation in HEK293 cells expressing TLR9, but stimulates NK cells to produce a significant level of IFN-a (unpublished data). Does this class of CpG DNA stimulate NK cells through another receptor or require a distinct cofactor to utilize TLR9 for the activation? Future studies on these mechanisms should prove fruitful ground for our growing understanding of CpG DNA/TLR9-mediated immune activation.

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