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## Cutting Edge: Role of Toll-Like Receptor 9 in CpG DNA-Induced Activation of Human Cells<sup>1</sup>

Fumihiko Takeshita,<sup>2,3</sup> Cynthia A. Leifer,<sup>2</sup> Ihsan Gursel, Ken J. Ishii, Saoko Takeshita, Mayda Gursel, and Dennis M. Klinman<sup>4</sup>

**Unmethylated CpG motifs present in bacterial DNA stimulate a rapid and robust innate immune response. Human cell lines and PBMC that recognize CpG DNA express membrane-bound human Toll-like receptor 9 (hTLR9). Cells that are not responsive to CpG DNA become responsive when transfected with hTLR9. Expression of hTLR9 dramatically increases uptake of CpG (but not control) DNA into endocytic vesicles. Upon cell stimulation, hTLR9 and CpG DNA are found in the same endocytic vesicles. Cells expressing hTLR9 are stimulated by CpG motifs that are active in primates but not rodents, suggesting that evolutionary divergence between TLR9 molecules underlies species-specific differences in the recognition of bacterial DNA. These findings indicate that hTLR9 plays a critical role in the CpG DNA-mediated activation of human cells. *The Journal of Immunology*, 2001, 167: 3555–3558.**

Members of the Toll-like receptor (TLR)<sup>5</sup> family respond to pathogen-associated molecular patterns expressed by a diverse group of infectious microorganisms (1), thereby triggering the host's innate immune system. TLRs contain an extracellular domain with leucine-rich repeats and an intracytoplasmic domain homologous to the IL-1R (2). Cellular activation by TLR proceeds through a signaling cascade involving myeloid differentiation marker 88, IL-1R-associated kinase (IRAK), TNFR-associated factor 6 (TRAF6), and NF- $\kappa$ B

translocation, culminating in the up-regulation of genes involved in host defense (2).

Unmethylated CpG motifs are present at a 20-fold higher frequency in bacterial than mammalian DNA, due to a combination of CpG suppression and CpG methylation (3, 4). CpG motifs trigger an innate immune response characterized by the activation of Ig-, cytokine-, and chemokine-secreting cells (3, 5). This response confers protection against a variety of intracellular pathogens consistent with unmethylated CpG motifs acting as pathogen-associated molecular patterns. Preclinical and clinical studies indicate that synthetic oligodeoxynucleotides (ODN) containing CpG motifs may have therapeutic value as immune adjuvants and anti-infectious agents (4, 6).

Recent studies indicate that TLR9 plays a critical role in the recognition of CpG motifs in mice. Dominant-negative (DN) versions of myeloid differentiation marker 88, IRAK, and TRAF6 inhibit CpG ODN-mediated cellular activation, and TLR9-knock-out mice fail to mount an immune response when stimulated with CpG ODN (7, 8). Despite 76% identity at the amino acid level between murine and human TLR9 (hTLR9) (7), the CpG motifs that are most active in mice have little effect on human cells, and vice versa (9). Moreover, the type of CpG motif expressed by various pathogens influences the type and magnitude of immune response elicited in different mammalian species (10). The present work examines whether CpG recognition in humans is also mediated by TLR9 and explores the nature of the receptor-ligand interaction.

### Materials and Methods

#### Reagents

Phosphorothioate ODNs were synthesized at the Center for Biologics Evaluation and Research core facility (Bethesda, MD). Sequences of the ODN (5'→3') were: K3 CpG ODN, ATCGACTCTCGAGCGTTCTC; K3-flip control ODN, ATGCACTCTGCAGGCTTCTC; K3-methyl, AT<sup>m</sup>CGAC TCT<sup>m</sup>CGAG<sup>m</sup>CGTTCTC; 2006, TCGTCGTTTGTGCTTTGTCTGTT (11); 1466, TCAACGTTGA; 1555, GCTAGACGTTAGCGT; and 1612, GCTAGATGTTAGCGT, where <sup>m</sup>C indicates a methyl cytosine. Cy3 was conjugated to the 5' end of some ODN. LPS was purchased from Sigma (St. Louis, MO). Human IFN- $\gamma$  was purchased from Life Technologies (Gaithersburg, MD).

#### Cells and cell cultures

Cell lines (obtained from American Type Culture Collection, Manassas, VA) were maintained in complete DMEM (10% FCS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 0.11 mg/ml sodium pyruvate, and 0.5 mM 2-ME). Elutriated monocytes and PBMC were obtained from the National Institutes of Health Blood Bank (Bethesda, MD).

#### Plasmid construction

Human TLR9 cDNA (the gift of Dr. B. Beutler, Scripps Research Institute, La Jolla, CA) (12) was inserted into pCIneo (Promega, Madison, WI).

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<sup>5</sup> Abbreviations used in this paper: TLR, Toll-like receptor; IRAK, IL-1R-associated kinase; TRAF6, TNFR-associated factor 6; ODN, oligodeoxynucleotides; hTLR9, human TLR9; DN, dominant negative; HA, hemagglutinin; ICD, intracellular domain; ECD, extracellular domain.

Human TLR9B (amino acids 58–1032), hTLR9  $\alpha$ 5 deletion mutant (1–1000), and hTLR9 intracellular domain (ICD) deletion mutant (1–860) were PCR generated from this cDNA. TLR9 (26–1032) was cloned into pDisplay (Invitrogen, Carlsbad, CA), which generated a hemagglutinin (HA) tag. PCR-amplified DN IRAK1 (1–96) and DN TRAF6 (287–523) were cloned into pFlagCMV4 (Sigma).

Cell transfection and luciferase assay

Cells ( $5 \times 10^4$ ) were transfected using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) plus 0.1  $\mu$ g p5xNF- $\kappa$ B-luc (Stratagene, La Jolla, CA), 0.1  $\mu$ g pSV- $\beta$ -galactosidase (Promega), and 0.2–0.8  $\mu$ g of various expression vectors for 18 h. Luciferase assays were performed as recommended by the manufacturer (Promega) after 24 h.  $\beta$ -Galactosidase activity was used to normalize the data.

RT-PCR

PCR (33–40 cycles)-amplified products from 1 to 5  $\mu$ g of reverse-transcribed RNA were visualized by ethidium bromide staining on agarose gels.

Confocal microscopy

Transfected 293T cells were treated with Cy3-labeled ODN for 10–120 min at 37°C. Cells were washed, fixed, permeabilized, and stained for HA-TLR9 protein using FITC-anti-HA Ab (clone 3F10; Roche Molecular Biochemicals). Subcellular localization of Cy3 and FITC signals were determined by confocal microscopy (LSM5 PASCAL; Carl Zeiss, Thornwood, NY).

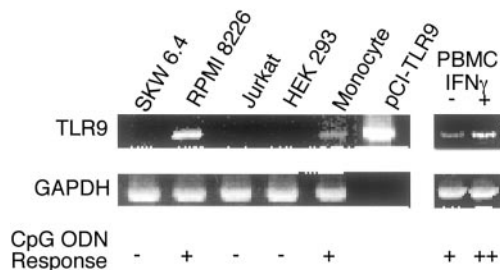
Results and Discussion

CpG responsiveness of human cells correlates with TLR9 mRNA expression

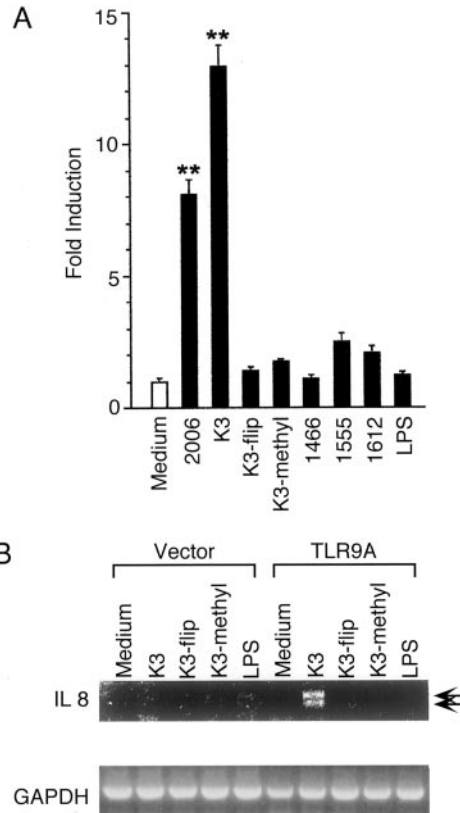
TLR9 mRNA was expressed in CpG-responsive human monocytes and RPMI 8226 cells, but not by unresponsive cells (such as Jurkat or 293; Fig. 1). Human PBMC constitutively expressed low levels of TLR9 mRNA. IFN- $\gamma$  treatment significantly increased both TLR9 mRNA expression and CpG DNA responsiveness of PBMC (Fig. 1 and data not shown). Therefore, TLR9 expression is a prerequisite for cell activation by CpG DNA, and factors that increase TLR9 mRNA levels also increase responsiveness to CpG DNA.

TLR9 confers responsiveness to CpG ODN that activate human cells

To examine the importance of TLR9 in the CpG-mediated activation of human cells, 293 cells were transiently cotransfected with a NF- $\kappa$ B-dependent luciferase reporter (p5xNF- $\kappa$ B-luc) plus hTLR9 (hereafter referred to as 293<sup>Trans</sup>). 293<sup>Trans</sup> stimulated with CpG ODN that activate human PBMC (9, 10) significantly increased NF- $\kappa$ B dependent luciferase activity (Fig. 2A). This stimulation was abrogated if the critical CpG motif was disrupted by



**FIGURE 1.** TLR9 mRNA expression correlates with CpG ODN responsiveness. RT-PCR specific for TLR9 or GAPDH (left panel) was performed on RNA isolated from various human cell lines, elutriated primary monocytes, and the pCIneo-TLR9 plasmid (no reverse transcription). Fresh human PBMC also expressed TLR9 mRNA (right panel). When stimulated with 1000 U/ml IFN- $\gamma$  for 18 h, the responsiveness of these PBMC and their levels of TLR9 mRNA both increased.



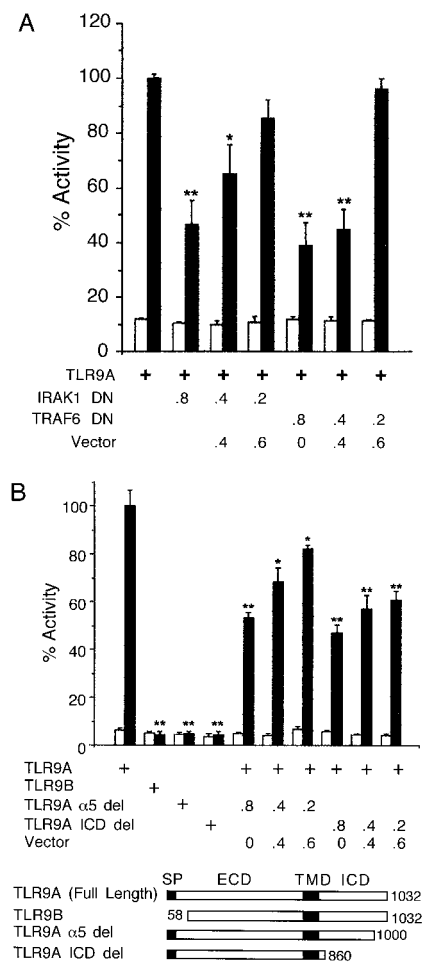
**FIGURE 2.** Expression of hTLR9 confers CpG ODN responsiveness. **A**, Luciferase activity of transfected cells 24 h after treatment with 1  $\mu$ M of the following ODN: human-stimulatory CpG ODN (2006 and K3), ODN in which the critical CpG dinucleotide was inverted or methylated (K3-flip and K3-methyl), murine-stimulatory CpG ODN (1466 and 1555), control ODN (1612), or 1  $\mu$ g/ml LPS. Results represent the mean  $\pm$  SEM of three to five independent experiments; \*,  $p \leq .01$ , and \*\*,  $p \leq .001$  compared with identically treated cells cultured in medium. **B**, IL-8 mRNA expression by 293 cells transfected with vector or TLR9 and stimulated for 24 h with 1  $\mu$ M CpG or control ODN as detected by PCR.

inversion or methylation (Fig. 2A). Moreover, CpG ODN known to trigger rodent but not primate cells (ODN 1555 and 1466) were uniformly inactive (Fig. 2A). The response of TLR9 was CpG specific, because 293<sup>Trans</sup> did not respond to LPS (Fig. 2A), and 293 cells transfected with TLR4 did not respond to CpG ODN (data not shown). These findings indicate that 1) TLR9 is involved in the recognition of human CpG motifs, and 2) differences in CpG recognition between species may reflect evolutionary divergence between TLR9 molecules.

The 293 cells transfected with TLR9 alone responded to CpG ODN by significantly increasing endogenous IL-8 mRNA expression (Fig. 2B). Control ODN had no effect on TLR9-transfected cells, and CpG ODN had no effect on mock-transfected cells.

IRAK1 and TRAF6 participate in the TLR9-dependent signaling cascade

DN forms of IRAK1 or TRAF6 inhibited TLR9-mediated luciferase activity in a dose-dependent manner (Fig. 3A). Although previous studies established that IRAK1 and TRAF6 were critical for CpG ODN-mediated cell signaling (8), current findings establish that their activation proceeds through TLR9 engagement. In contrast, cofactors known to stabilize cell membrane expression of other members of the TLR family, such as MD1 and MD2 (13), did not influence CpG ODN-mediated activation of 293<sup>Trans</sup> (data not shown).



**FIGURE 3.** CpG ODN-mediated TLR9 signaling. *A*, The 293 cells were cotransfected with p5xNF- $\kappa$ B-luc, TLR9, and varying amounts of DN IRAK1 or TRAF6 plus vector to yield a constant 0.8  $\mu$ g DNA/reaction. Data show luciferase activity 24 h after incubation in medium ( $\square$ ) or 1  $\mu$ M CpG ODN ( $\blacksquare$ ). *B*, Same as in *A* except 293 cells were cotransfected with p5xNF- $\kappa$ B-luc, TLR9/B, and/or 0.2–0.8  $\mu$ g of ICD-deleted TLR9 mutants ( $\alpha$ 5 del or ICD del). The diagram shows TLR9 and truncation mutants. SP, signal peptide; TMD, transmembrane domain. Results represent the mean  $\pm$  SEM of three independent experiments; \*,  $p \leq 0.05$ , and \*\*,  $p \leq 0.01$  compared with cells transfected with TLR9 alone.

*Contribution of intracellular and extracellular domains to cell signaling*

To identify those regions of the TLR9 molecule critical to cell signaling, deletion mutants were generated (Fig. 3*B*). Cells transfected with TLR9B (lacking the NH<sub>2</sub>-portion of TLR9; Ref. 12) did not respond to CpG ODN (Fig. 3*B*). As expected, eliminating the entire ICD also abrogated CpG ODN-mediated NF- $\kappa$ B activation (Fig. 3*B*). Interestingly, a TLR9 construct lacking only the C-terminal 32 amino acids of the ICD was also inactive, suggesting that this region plays a critical role in cell signaling.

Because eliminating the extracellular domain (ECD) can constitutively activate TLRs (14), 293 cells were cotransfected with TLR9 plus an ICD deletion mutant to examine the contribution of the ECD to TLR9-mediated signaling/activation. CpG ODN-dependent cellular activation was suppressed in a dose-dependent fashion by both the  $\alpha$ 5 and ICD deletion mutants (Fig. 3*B*). This effect was most likely mediated by ECD interactions, because co-transfection did not alter TLR9 mRNA expression (data not shown). Thus, similar to other members of the TLR family, TLR9

signaling appears to involve the generation of multimers through ECD interactions (15, 16).

*Cellular localization of CpG ODN and TLR9*

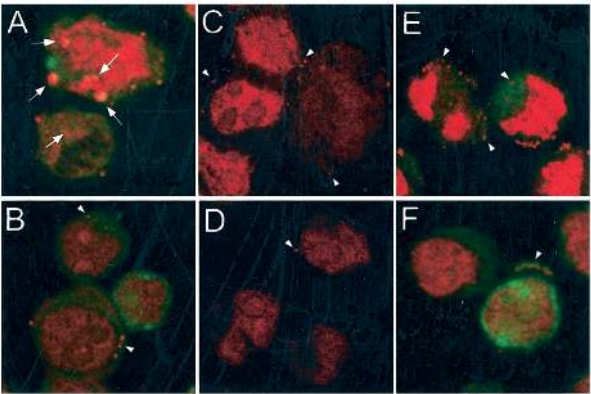
Several members of the TLR family are expressed on the plasma membrane (13, 17). Signaling through TLR2 involves the redistribution of the receptor from the membrane into phagosomal vesicles (17). Although uptake by acidified endocytic vesicles may be required for CpG-mediated signaling (18, 19), recent reports suggest that CpG ODN can bind to the plasma membrane and need not be internalized to trigger (20).

To examine the relationship between CpG binding, endocytosis, and signaling, a TLR9 construct encoding a HA tag (HA-TLR9) was generated. Cells transfected with HA-TLR9 specifically bound FITC-anti-HA Ab (Fig. 4*A*) and activated NF- $\kappa$ B in response to CpG but not control ODN (data not shown). Cell surface staining of HA-TLR9 transfectants showed that a fraction of the TLR9 is on the cell surface (data not shown). Because transfected 293T cells over-express HA-TLR9, the location of this molecule under physiologic conditions requires further study.

Cells expressing HA-TLR9 were incubated with Cy3-labeled ODN. CpG ODN initially associated with the cell surface, began to form vesicles near the surface, and entered the nucleus of HA-TLR9-transfected cells within 10 min (data not shown). By 2 h, the size and number of CpG ODN-containing vesicles had increased, and the vesicles relocated from near the plasma membrane to intracellular regions (Fig. 4*A*). In some cases, both hTLR9 and CpG ODN were colocalized within the same endocytic vesicle (Fig. 4*A*).

Control (non-CpG) ODN also rapidly gained access to the nucleus and formed small vesicles near the surface of HA-TLR9-transfected cells. However, these vesicles did not change in size or number over time, nor did they relocate within the cell (Fig. 4*B*). Similarly, ODN reached the nucleus of cells transfected with vector alone, but induced minimal vesicle formation (Fig. 4, *C* and *D*). Thus, cells that lack TLR9 can internalize DNA in a sequence-nonspecific manner, but TLR9 enhances vesicular uptake, vesicle relocation, and cellular activation in the presence of CpG motifs.

To verify these conclusions, 293T cells were transiently transfected with HA-TLR9 ICD deletion mutant, a mutant TLR9 lacking the cytoplasmic tail. This mutant does not signal, instead acting



**FIGURE 4.** Cellular localization of TLR9 and CpG ODN. The 293T cells were transfected with HA-TLR9 (*A* and *B*), vector (*C* and *D*), or HA-TLR9 ICD deletion mutant (*E* and *F*) and treated for 2 h with Cy3-CpG ODN (red; *A*, *C*, and *E*) or Cy3-control ODN (*B*, *D*, and *F*). Cells were then fixed, stained with FITC-conjugated anti-HA Ab (green), and analyzed by confocal microscopy. Arrows show larger yellow/orange vesicles, indicating colocalization; and arrowheads show smaller, more peripheral red vesicles. Representative sections from one of six independent experiments (magnification,  $\times 1000$ ) are shown.



as a DN when coexpressed with hTLR9 (Fig. 3). Similar to vector-transfected cells, ODN gained access to the nucleus but formed only small peripheral vesicles in cells transfected with the HA-TLR9 ICD deletion mutant (Fig. 4, *E* and *F*). Prolonged incubation did not increase in the number or size of CpG-containing vesicles and rarely triggered their relocation. Thus, cellular activation through TLR9 was linked to enhanced vesicular uptake of CpG ODN.

### Conclusions

This work provides three fundamental insights into the role of hTLR9 in CpG-mediated activation of human cells. First, expression of TLR9 is a prerequisite for CpG ODN responsiveness. This supports and extends observations in mice that TLR9 plays a critical role in CpG recognition (7). Our results demonstrate that hTLR9 is a cell surface receptor expressed by CpG-responsive cells, and that hTLR9 transfection confers CpG reactivity to cells that are otherwise nonresponsive. Second, hTLR9 enhances vesicular uptake of CpG but not control ODN. In some cases, TLR9 and CpG ODN colocalize within the same vesicles. Although ODN enters cells that lack TLR9 (or express signal-defective TLR9 mutants), this uptake is sequence independent and does not influence vesicle formation. Together, these observations suggest that vesicular uptake of CpG ODN is associated with cell signaling. Third, the recognition of CpG DNA by hTLR9 is exquisitely sequence specific. Eliminating the CpG dinucleotide by inversion or methylation abrogates responsiveness. Moreover, the CpG flanking region determines whether an ODN will activate human cells, and concomitantly, whether it will trigger through hTLR9. These findings suggest that species-specific differences in the recognition of bacterial DNA evolved through diversification of TLR9.

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