Article

Specific siRNA Downregulated TLR9 and Altered Cytokine Expression Pattern in Macrophage after CpG DNA Stimulation

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Bacterial CpG DNA or synthetic oligonucleotides (ODNs) that contain unmethylated CpG motifs (CpG ODN) can directly activate antigen-presenting cells (APCs) to secrete various cytokines through the intracellular receptor TLR9. Cytokine profiles elicited by the actions of stimulatory CpG DNA on TLR9 expressed APCs are crucial to the subsequent immune responses. To date, cytokine profiles in APCs upon CpG ODN stimulation *in vitro* are not fully investigated. In the present study, vector-based siRNA was used to downregulate TLR9 expression. Cytokine profiles were observed in murine macrophage cell line RAW264.7 transfected with TLR9-siRNA plasmid upon CpG ODN stimulation. We found that not all the cytokine expressions by the macrophage were decreased while TLR9 was downregulated. IL-12, TNF-α, IFN-γ and IL-1β expressions were significantly decreased, but IL-6, IFN-β and IL-10 expressions were not affected. Interestingly, the level of IFN-α was even increased. This alteration of cytokines produced by TLR9-downregulated APCs upon CpG ODN stimulation might indicate that the role of CpG DNA is more complicated in the pathogenesis and prevention of diseases. *Cellular & Molecular Immunology*. 2005;2(2):130-135.

Key Words: TLR9, RNA interference, RAW264.7, CpG DNA

Introduction

Pathogens including bacteria, fungi and viruses can release unmethylated CpG DNA when the host cells are infected. The binding of the CpG DNA to its functional receptor TLR9 expressed in antigen-presenting cells (APCs) could lead to the activation of APCs and the production of cytokines, including type I interferon (IFN-α), tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-12, IFN-γ, IL-6 and IL-18 (1-14). Cytokine profiles induced in this process are considered to be crucial to T cell activation and later immune response. Cells deficient for TLR9 are unresponsive to CpG DNA and cytokine productions are virtually abrogated. However, there are few reports on the changes of the cytokine profiles if TLR9 is downregulated but not completely knocked out. In the present study, to reduce TLR9 expression, vector-based small interference RNA (siRNA) was constructed and applied to transfect a murine macrophage cell line RAW264.7. The expression level of TLR9 in the macrophage stimulated with CpG ODN *in vitro* was examined by both RT-PCR and Western Blot analysis. We observed that not all the cytokine levels were decreased, IL-12, TNF-α, IFN-γ and IL-1β expression were obviously decreased, but the expression levels of IL-6, IFN-β and IL-10 were not influenced. Beyond our expectation, the level of IFN-α was even increased. The possible interpretations for these findings were discussed. These altered cytokine expression patterns suggest that the role of CpG DNA is more complicated in the pathogenesis and prevention of diseases.

Materials and Methods

Reagents

psiRNA-hH1GFPzeoG2 expression vector containing human H1 promoter and GFP reporter gene was purchased from Invivogen (USA). *E.coli* GT116 was maintained in our laboratory. T4 DNA ligase and restrictive endonuclease Bbs I were products of BD company (USA). Trizol regent and M-MLV reverse transcriptase were from MBI (Shanghai). jetPEI was purchased from Polyplus transfection company (France). Cytokines ELISA kits were products of R&D Systems, Inc. (USA). Rabbit anti-mouse TLR9 polyclonal antibody was from eBioscience (USA).

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Synthetic ODN

The hairpin oligonucleotides of mouse TLR9 (No.1: 5'-ACC TCG TCC TAT AAC CTC ATT GTC AAT CAA GAG TTG ACA ATG AGG TTA TAG GAC TT-3'; No.2: 5'-ACC TCG TTC AGT GAG CTA CCA CAG TTT CAA GAG AAC TGT GGT AGC TCA CTG AAC TT-3'; No.3: 5'-ACC TCG CCT CCG AGA CAA CTA CCT ATT CAA GAG ATA GGT AGT TGT CTC GGA GGC TT-3') were synthesized by Shanghai SBS Genetech Co., Ltd (Shanghai). The sequences of phosphorothioate-stabilized CpG ODN 1668 are 5'-TCC ATG ACG TTC CTG ATG CT-3', a proven activator of murine immune cells as described previously (14).

Cell culture

A murine RAW264.7 macrophage cell line was maintained in culture medium consisting of DMEM supplemented with 10% heat-inactivated FCS (GIBCO) containing 2 mM glutamine (Sigma), 100 IU/ml penicillin G, and 100 mg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator.

Construction of phH1GFP-TLR9 specific siRNA expression vector

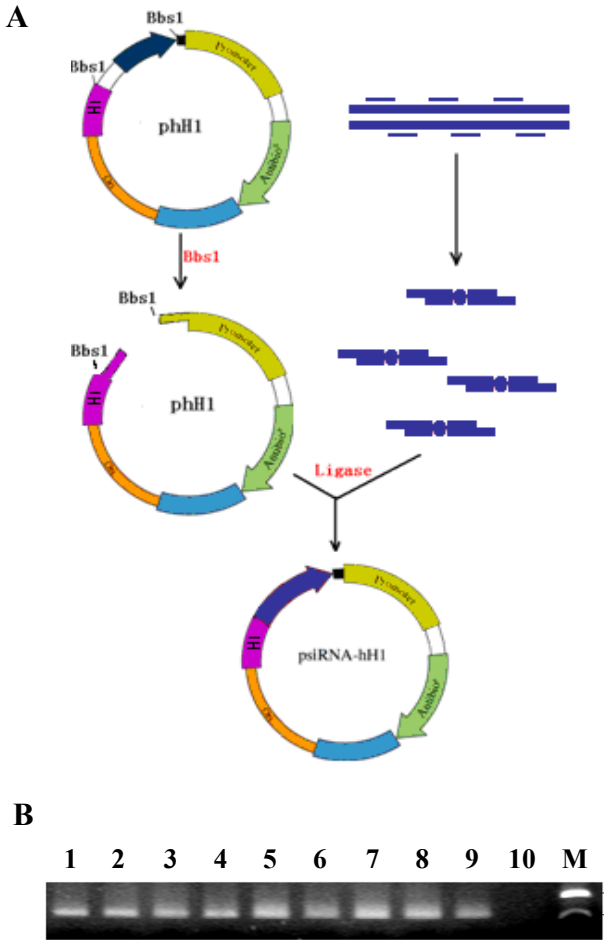
According to the manufacturer's instruction (Invivogen), 3 different sense and antisense DNA oligonucleotides were synthesized for *in vitro* transcription to generate TLR9 sense and antisense siRNA. After hybridization, the purified double-stranded siRNAs were cloned into Bbs I sites of phH1GFP. The correctness of three constructs (phH1GFP-TLR9) was confirmed by nucleotide sequencing (Shanghai United Gene Holdings, LTD).

Transfection of RAW264.7 cells with TLR9 siRNA

RAW264.7 cells were transfected with 1 μg of phH1GFP-TLR9 or phH1GFP plasmids respectively. Transfection was carried out using jetPEI according to the manufacturer's instruction. After transfection for 48 h, Zocin (BD Biosciences, 200 μg/ml final concentration) was added to the culture medium for selection. The resultant cell clones were screened by RT-PCR and Western blot.

RT-PCR

Total RNAs were extracted using Trizol reagents according to the manufacturer's instruction. Complementary DNA was generated using Oligo (dT) (Promega) and M-MLV reverse transcriptase (MBI). For the PCR reaction, 4 μl of cDNA from each synthesis were added to 50 μl of reaction mixture containing 5 μl of 10 × PCR buffer, 1 μl of 10 mM dNTP mix, 2 μl of 25 mM $MgCl_2$, 0.5 μl (5 U/μl) of Taq DNA polymerase (Biostar) and 35.5 μl ddH_2O. An amount of 20 pmol each pair of TLR9 specific primers (sense: 5'-GCA CAG GAG CGG TGA AGG T-3'; antisense: 5'-GCA GGG GTG CTC AGT GGA G-3') was added and the homekeeping gene of GAPDH (sense: 5'-CTG CAC CAC CAA CTG CTG CTT AG-3'; antisense: 5'-GTC TGG GAT GGA AAT AAT TGT GA-3') was used as control. The mixtures were denatured at 94°C for 30 s, annealed at 56°C for 30 s and extended at 72°C for 45 s for 30 cycles. PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. TLR9 transcript levels were normalized by GAPDH.



Figures 1. Construction and identification of phH1GFP-TLR9 plasmids. (A) Physical map of phH1GFP-TLR9 siRNA eukaryotic expression plasmid. (B) PCR analysis of the recombinant phH1GFP-TLR9 siRNA plasmid. PCR analysis was performed as described in "Materials and Methods" to prove the correctness of inserted fragments.

Western blot

RAW264.7 cells stably transfected with TLR9 siRNA vector and the untransfected cells were lysed using lysis buffer for 15 min at 4°C. The cell lysates were subjected to SDS-PAGE and then transferred onto nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST (Triton X-100 0.5%, Tris-buffered saline), incubated with rabbit anti-mouse TLR9 polyclonal antibody (1:500) for 2 h, washed with TBST, and stained with peroxidase-conjugated IgG secondary antibody (1:1,000). Immunoreactivity was visualized by enhanced chemiluminescence (ECL kit, Santa Cruz).

Cytokine assay

Cytokines in the supernatants of cultured RAW264.7 cells were measured by ELISA as recommended by manufacturer. Briefly, supernatants were collected 48 h after CpG ODN (6 μg/ml) stimulation. Capture antibodies were coated overnight at 4°C on Immunlon 96-well plates at a concentration of 3 μg/ml in 0.1 M phosphate buffer (pH 9.0). Plates were washed 3 times, and then samples were added in duplicate and incubated at room temperature for 2 h. Biotinylated detection antibodies were added and incubated for an additional 2 h at room temperature. Avidin-conjugated horseradish peroxidase (EB, USA) was added for 30 min incubation at room temperature followed by TMB substrate (0.015% 3,3',5, 5'-tetramethylbenzidine, 0.01% H_2O_2 in 0.1 M citrate buffer, pH 4.0) until color development. Three PBS washes were performed between steps and plates were read at OD_{380} using an automated microtiter plate reader (Southern Biotech, USA).

Statistical analysis

The statistical significance of the results was evaluated using the Mann-Whitney U-test. Student's *t* test was used when appropriate. All data were expressed as mean ± standard deviation and $p < 0.05$ was considered significantly different.

Results

Construction and identification of siRNA plasmids containing TLR9 specific hairpin structure

Three hairpin TLR9 siRNAs were designed according to the murine TLR9 gene sequence from GenBank (Accession No. NM_031178). Using the DNA recombination technique, the synthetic oligonucleotides containing the target sequences were inserted into the Bbs I sites of phH1GFP parental vectors (Figure 1A). Nine clones were identified to contain the inserted siRNA fragments amplified by PCR with TLR9-specific primers (Figure 1B). Three positive clones were randomly selected for further studies.

Detection of TLR9 expression of RAW264.7 cells transfected with siRNA plasmid

To observe the interference effect of the constructs, RAW264.7 cells were transfected with three different phH1GFP-TLR9 siRNA plasmids, respectively. RAW264.7 cells transfected with phH1GFP parental plasmid or without any treatment were used as negative controls. After transfection, TLR9 expression levels were examined by RT-PCR and Western blot. As shown in Figure 2, TLR9 expression both at gene (Figure 2A) and protein levels (Figure 2B) in the phH1GFP-TLR9 siRNA-transfected cells were significantly decreased compared with the negative controls. TLR9 mRNA levels in transfected cells were decreased 44.6%, 56.9% and 64.6% when each of siRNA was used. The decreased protein level was 34.7%, 48.5% and 80.2% in the transfected cells with each of the three TLR9-siRNA plasmids. Both the TLR9 mRNA level and the protein level were decreased most obviously in No.3 siRNA transfected cells. In contrast, TLR9 expression was not influenced in both untransfected cells and the parental plasmid transfected cells.

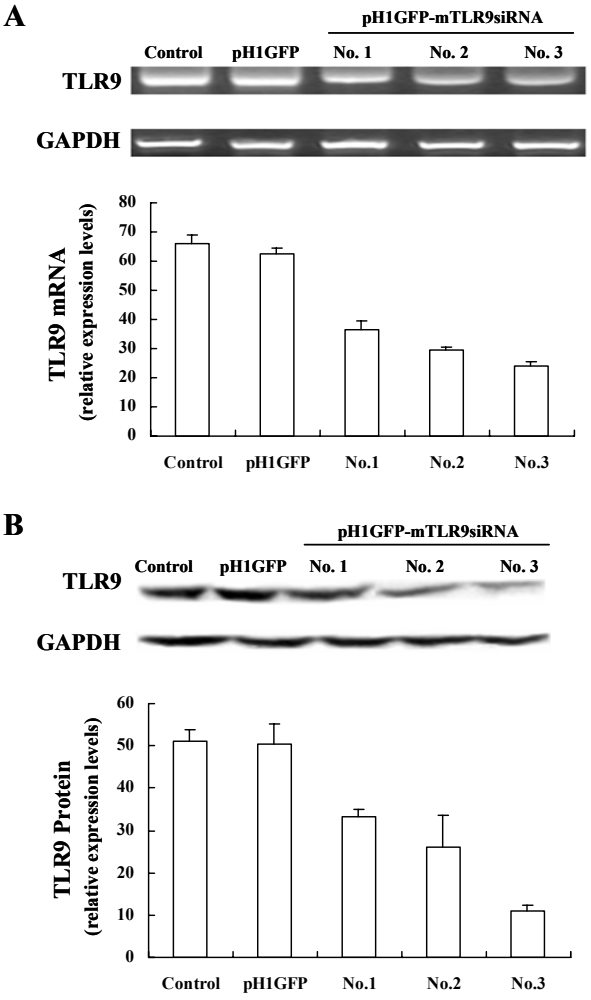


Figure 2. siRNA downregulates TLR9 expression. (A) After transfection of TLR9 siRNA vector into RAW264.7, TLR9 mRNA was examined by RT-PCR and normalized by GAPDH levels. TLR9 mRNA level was decreased 44.6%, 56.9% and 64.6% by No.1, No.2 and No.3 siRNA. (B) TLR9 protein levels were tested by Western blot and normalized by GAPDH levels as described in "Materials and Methods". TLR9 protein level was decreased 34.7%, 48.5% and 80.2% by the transfected cells with each TLR9-siRNA plasmid.

Characteristics of three siRNAs

Three plasmids had different interference efficiency, so their inserted siRNA sequences were sequenced (Figure 3). By using e-analysis with Mfold software, H-b index and GC content for each siRNA was calculated. As shown in Table 1, H-b index of No.3 sequence is the lowest and GC content was 52.4% (percentage of GC content in 21 nucleotides), which was close to 50%. Its 5'-GC content was the richest

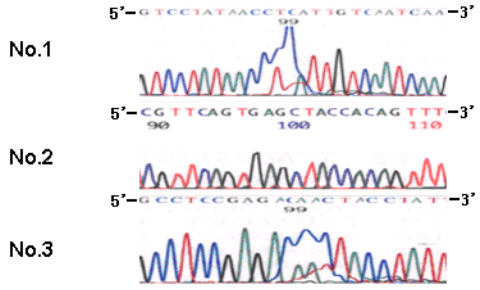


Figure 3. Identification of inserted fragments by sequencing.

(percentage of GC in the first seven nucleotides). So, No.3 siRNA sequence was most consistent with the principles of siRNA designing.

Altered expression pattern of cytokines induced by CpG DNA-stimulated RAW264.7 cells pretreated with TLR9 specific siRNA

RAW264.7 cells transfected with No.3 TLR9-siRNA palsmid were stimulated by CpG ODN (1 μM). The cell supernatants were harvested and cytokines levels were determined 24 h-post stimulation. As shown in Figure 4, compared with untransfected group and parental plasmid-transfected group, the expression levels of IL-12, TNF-α and IFN-γ as well as IL-1β in the interference group were significantly decreased, whereas IL-6, IFN-β and IL-10 levels were not affected by siRNA plasmid. To our surprise, the expression levels of IFN-α were increased in the interference group. These altered cytokine expression patterns were summarized in Figure 5.

Discussion

Although three approaches including gene knockout, antisense technique and RNA interference are often used to eliminate or suppress target gene expression, RNAi has been recently shown to be a most powerful approach for analyzing gene function (15-17). RNAi is the technique of post-transcriptional dsRNA-dependent gene silencing. Cellular delivery of synthetic small interfering RNA duplexes (siRNA) or introduction of siRNA by plasmids is now widely used to disrupt the activity of cellular genes homologous in sequence to the introduced dsRNA (18). In general, siRNA duplexes (generally 19-21 base pairs) are thought to be short enough to bypass general dsRNA-induced nonspecific effects in vertebrate cells (19，20).

Researchers (21) have now developed methods for RNAi in which siRNAs are synthesized in mammalian cells from expression vectors. The use of DNA plasmids can facilitate RNAi in cell types that are difficult to transfect with *in vitro*-synthesized siRNAs, as well as allow the generation of cell lines that stably express siRNAs. Another potential application of vector-based RNAi in mammals is combinatorial inhibition of genes in somatic cells. The ability to inhibit two or more genes simultaneously using hairpin siRNA expression vectors, either in cultured cells or in whole animals, would facilitate analysis of multiple gene families with overlapping functions in vertebrates (21). At present, the design of siRNAs and hairpin siRNAs is an empirical process, because the molecular mechanisms underlying RNAi are not yet sufficiently understood to allow rational design of siRNAs. In the present study, we designed three TLR9 siRNAs and inserted them into phH1GFP vectors. We found that No.3 TLR9-specific siRNA eukaryotic expression plasmid worked most efficiently since TLR9 expression in macrophage both at mRNA level and protein level was significantly decreased. Therefore, the most efficient TLR9 siRNA sequence was GCC TCC GAG ACA ACT ACC TAT under our experimental condition. After analyzing the properties of TLR9-siRNA, we found that GC content of No.3 siRNA sequence was closest to 50% and GC was richest at the 5' end. These data conformed to the principles described previously. In addition, we examined the H-b index was relatively low in No.3 siRNA. This was consistent with the report that siRNA efficiency was correlated with the hydrogen bond index (H-b index). The higher H-b index, the lower siRNA efficacy (22). From Table 1, we noticed these three siRNA targeted different positions in TLR9 mRNA, it is probably that the local structure of the targeted regions correlated with the siRNA efficacy.

To further observe the RAW264.7 cells transfected with TLR9-siRNA palsmid in response to CpG ODN, several cytokine levels were tested. We observed that IL-12, TNF-α, IFN-γ and IL-1β levels were significantly decreased. This was consistent with the findings that interactions between CpG DNA and TLR9 activate APCs through the Toll/IL-1-receptor signaling pathway to produce Th1-polarizing cytokines (23-26), such as IL-12 and IL-18 and IFN-γ as well as pro-inflammatory cytokines including TNF-α, IL-6

Table 1. Characteristics of TLR9 siRNA sequences

No.	Sequence	Position in TLR9 mRNA	H-b index	5'-GC	GC
1	GTCCTATAACCTCATTGTCAA	787-807	3.2	14.3%	38.1%
2	GTTCAGTGAGCTACCACAGTT	1729-1749	27.1	14.3%	47.7%
3	GCCTCCGAGACAACTACCTAT	2085-2105	20.4	28.5%	52.4%

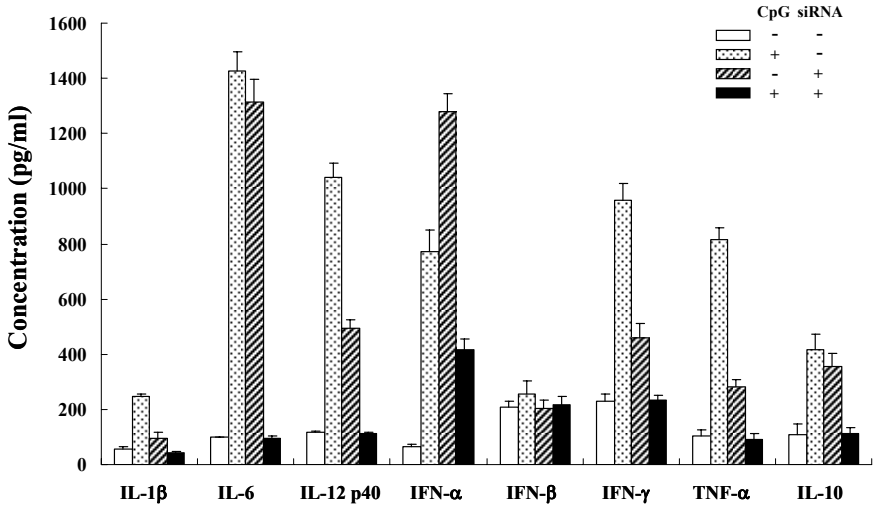


Figure 4. Cytokine profiles of TLR9-siRNA transfected macrophage. RAW264.7 cells transfected with or without TLR9 siRNA palsimd were stimulated *in vitro* with synthetic CpG ODN for 24 h. After stimulation, the cell supernatants were harvested and several cytokines levels were determined by ELISA. Data shown here are representative of two separate experiments. "-" indicates "without" and +" indicates "with". $*p < 0.05$, $**p < 0.01$.

and type I IFN. Although TLR9-mediated activation induces generation of pro-inflammatory cytokines including TNF-α, IL-6 and type I IFN, our data showed that IL-6, IFN-β and IL-10 levels were not affected. These results probably were attributed to the effects of plasmid backbone or the transfection reagents.

To our surprise, the expression levels of IFN-α were increased after CpG stimulation. There might be several explanations for this observation. Recently, it was reported that some of the synthetic siRNA sequences show vigorous IFN-α response. Researchers illustrated (27) a candidate for mediating these responses to dsRNA is the RNA-dependent protein kinase (PKR). But the pathways of activation by RNA are not fully revealed. Alternatively, we used nucleaseresistant phosphorothioate (PS)-modified CpG-ODN instead of phosphodiester ODN to increase the stability against degradation and enhance the stimulatory effects (28). However, PS-backbone also induces some sequence-independent stimulation (29), which might be served as another explanation.

Transfection of cells with siRNA can result in PKR-dependent type I IFN response (30). Furthermore, siRNA can lead to the induction of type I IFN (IFN-α) responses through TLR3 expressed in cell lines, including HEK293 cells (31, 32). Inconsistent with our results, it has also been reported that synthetic siRNA does not induce a type I IFN response in cell lines (33). This phenomenon may be more complicated than previously described, which need to be further investigated.

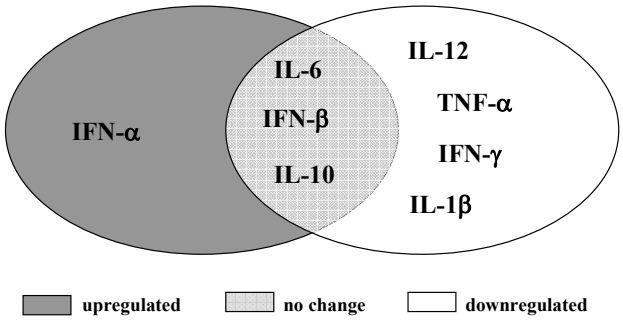


Figure 5. Summary of changes of cytokine expression in RAW264.7 after TLR9 expression was downregulated. Dark area shows the upregulated cytokine is IFN-α. Blank area shows downregulated cytokines are IL-12, TNF-α, IFN-γ and IL-1β. Grey area shows those cytokines expression levels are not affected.

Acknowledgements

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