A Single Nucleotide Polymorphism in 3'-Untranslated Region Contributes to the Regulation of Toll-Like Receptor 4 Translation

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Running title: Translational regulation by a 3'-UTR transversion in *TLR4*

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Capsule

Background: Genetic variation of SNP rs11536889 in 3'-UTR of *TLR4* is implicated in certain diseases, including periodontitis, gastric atrophy, and prostate cancer.

Results: The G allele of rs11536889 inhibited translation, but not transcription, of TLR4.

Conclusion: Genetic variation of rs11536889 regulates TLR4 expression.

Significance: Polymorphism in rs11536889 could be an excellent genetic marker for the diseases caused by TLR4-ligands.

Abstract

We have previously shown that a single nucleotide polymorphism (SNP) rs11536889 in the 3'-untranslated region (UTR) of *TLR4* was associated with periodontitis. In this study, the effects of this SNP on Toll-like receptor (TLR) 4 expression were investigated. Monocytes from subjects with the C/C genotype expressed higher levels of TLR4 on their surfaces than those from subjects with the other genotypes. Peripheral blood mononuclear cells (PBMCs) from the C/C and G/C subjects secreted higher levels of IL-8 in response to lipopolysaccharide (LPS), a TLR4 ligand, than the cells from the G/G subjects. However, there was no significant difference in TLR4 mRNA levels in PBMCs from the subjects with each genotype. Following stimulation with Pam_3CSK_4, TLR4 mRNA levels increased in PBMCs from both the C/C and G/G subjects, whereas TLR4 protein levels increased in PBMCs from the C/C, but not G/G subjects. Transient transfection of a series of chimeric luciferase constructs revealed that a fragment of 3'-UTR containing rs11536889 G allele, but not C allele, suppressed luciferase activity induced by LPS or IL-6. Two microRNAs, hsa-miR-1236 and hsa-miR-642a, were predicted to bind to rs11536889 G allele. Inhibition of these microRNAs reversed the suppressed luciferase activity. These microRNA inhibitors also up-regulated endogenous TLR4 protein on THP-1 cells (the G/G genotype) following LPS stimulation. Furthermore, mutant microRNAs that bind to the C allele inhibited the luciferase activity of the construct containing the C allele. These results indicate that genetic variation of rs11536889 contributes to translational

regulation of TLR4, possibly by binding to microRNAs.

Introduction

Toll-like receptor (TLR) 4 is a pattern recognition receptor that plays an essential role in the recognition of Gram-negative bacterial lipopolysaccharides (LPS) (1). The binding of LPS to myeloid differentiation factor 2 (MD-2)-TLR4 complex induces the formation of an M-shaped receptor multimer and leads to the assembly of the MyD88–IRAK4–IRAK2 complex (2). Formation of these Myddosome complexes leads to nuclear factor-κB and mitogen-activated protein kinase activation, which induces pro-inflammatory cytokines (3,4). TLR4 also recruits TIR-containing adaptor inducing interferon-β (TRIF) and TRIF-related adapter molecule in endosomes, which activates transcription factor interferon regulatory factor 3 and induces genes encoding type I interferons (5). TLR4 is reported to recognize exogenous ligands other than LPS, such as the fusion protein of the respiratory syncytial virus (RSV) and the envelope protein of the mouse mammary tumor virus (6). In addition, endogenous molecules such as heat-shock proteins, β-defensin-2, high mobility group box 1 protein, and fibronectin are reported to interact with TLR4 (7).

Inherited defects in TLR4 signaling have been described in humans and are associated with greater susceptibility to bacterial infections (8). Two missense mutations in *TLR4* (D299G and T399I) have been reported to be associated with endotoxin hyporesponsiveness to inhaled LPS (9). This report was followed by a series of studies investigating the potential impact of these SNPs on the incidence and course of infectious diseases (10), such as septic shock with Gram-negative bacterial infection (11) and severe RSV bronchiolitis in infants (12). Several papers reported the importance of these SNPs for incidences of cardiovascular diseases as well, which possibly reflects the inflammatory nature of atherosclerosis (13). Despite the accumulating reports concerning the susceptibility to such diseases, the resent studies using primary cells isolated from individuals bearing the mutation have indicated that the D299G/T399I haplotype has little or no effect on responsiveness to LPS (14).

We tried to determine whether D299G and T399I mutations in *TLR4* were associated with periodontitis because TLR4 is considered to be involved in the pathogenesis of periodontal diseases (15). However, two missense mutations in *TLR4* (D299G and T399I) are very rare in the Japanese population (16). We searched for single nucleotide polymorphisms (SNPs) in *TLR4* because it is possible that other SNPs are associated with periodontitis in the Japanese population. We found 4 SNPs in the exons of *TLR4*, although none of them resulted in amino acid substitutions. One SNP in the 3'-untranslated region (UTR) of *TLR4*, National Center for Biotechnology Information ID; rs11536889, was associated with moderate to severe periodontitis in Japanese subjects. The C/C genotype of rs11536889 was significantly higher in both the moderate and the severe periodontitis group, than in the control group (16). Another large study analyzing eight TLR4 SNPs in 1,383 prostate cancer patients and 780 age-matched controls in Sweden revealed that the G/C or C/C genotype of rs11536889 increased prostate cancer risk by 4.9% (17). Later, Hishida *et al*. found that the G/C or C/C genotype was

associated with severe gastric atrophy in *H. pylori* seropositive Japanese subjects (18). Zhou *et al.* found that the C/C genotype was significantly associated with hepatitis type B virus (HBV) recurrence after liver transplantation (19). Miedema *et al*. found that this SNP was associated with an increased risk of developing chemotherapy-induced neutropenia in children with acute lymphoblastic leukemia (20). These findings suggest that the genetic variation of rs11536889 may have influence on human inflammatory and/or malignant diseases. Since rs11536889 is located in the 3'-UTR of *TLR4*, it should not have a direct influence on the conformation of the TLR4 protein molecule. However, since SNPs in introns and/or UTRs could influence transcription and/or translation (21-24), the genetic variation of rs11536889 may have a direct effect on mRNA stability or translation efficiency.

The aim of this study was to determine whether the rs11536889 polymorphism is associated with expression or function of TLR4. The expression levels of TLR4 mRNA and protein in peripheral blood from C/C subjects were compared with those from G/C and G/G subjects. The regulatory effects of TLR4 3'-UTR on gene expression was measured by a luciferase reporter assay using chimeric constructs containing a small fragment of 3'-UTR containing rs11536889 in THP-1 cells. MicroRNA (miRNA) are approximately 22 nucleotide non-coding RNAs that guide the RNA-induced silencing complex to the 3'-UTR of mRNA targets, leading to the inhibition of translation of the target mRNA (25). The possible effects of miRNA were examined using miRNA inhibitors and miRNA mimics. We show here the regulation of TLR4 expression by rs11536889 polymorphism.

EXPERIMENTAL PROCEDURES

Subjects- Twelve C/C subjects and their age- and sex-matched G/C and G/G controls whose genotype were analyzed in our previous study (16) were enrolled in the flow cytometry study. Another 100 subjects who visited Nagasaki University Hospital were screened for rs11536889 genotypes; C/C subjects and their controls were enrolled in the enzyme-linked immunosorbent assay (ELISA) and quantitative reverse transcription PCR (qRT-PCR) study. All of the subjects were Japanese. Individuals with pregnancy, diabetes mellitus, malignancy, immunodeficiencies, or infectious diseases such as acquired immune deficiency syndrome and adult T cell leukemia, were excluded. The gender and mean age of the subjects are shown in Table 1. There was no significant difference in age and male/female ratio between each genotype group. Written informed consent was obtained from all of the participants in this study. The experimental protocol was approved by the Ethics Committee in Nagasaki University.

Genotyping- Buccal mucosal cells were taken from 100 newly enrolled subjects in this study. DNA was extracted with a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). The *TLR4* rs11536889 polymorphism was genotyped by PCR with confronting two-pair primers (PCR-CTPP) according to the procedure by Hishida *et al*. (18). The sequences of the primers were described previously (18). Amplification conditions were 10 minutes of initial denaturation at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 65 °C, and 1 min at 72 °C, then a 5-min final extension at 72 °C. The amplified DNA was visualized on a 2% agarose gel with ethidium

bromide staining. The amplified DNA was 184 bp for G allele, 256 bp for C allele, and 397 bp for common band.

Flow Cytometric Analysis of Freshly Isolated Peripheral Blood Monocytes- Peripheral blood mononuclear cells (PBMC) were isolated from 12 C/C subjects, and their controls with the G/C and G/G genotypes, and separated by gradient centrifugation of heparinized blood using endotoxin-free Ficoll-Paque (GE-Healthcare Bio-Sciences, Uppsala, Sweden) according to manufacturer's protocol. The cells were double-stained with anti-CD14-PE (clone UCHM-1, Sigma-Aldrich, St. Louis, MO) and anti-TLR2-FITC (clone TL2.1, eBioscience, San Diego, CA), anti-TLR4-FITC, (clone HTA125, Imgenex, San Diego, CA), or isotype-matched control-FITC (clone MOPC141, Sigma-Aldrich) monoclonal antibodies. Following washing in 1% fetal bovine serum (FBS) (Moregate Biotech, Bulimba, Australia) in phosphate buffered saline, the cells were resuspended in 2% formaldehyde for 30 min and washed again. The cells were resuspended in the solution consisting of 10% Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich) and 90% FBS, and stored at –80 °C for batch analysis. On the day of analysis, all cells were thawed and washed in 1% FBS and expression levels of TLR2 and TLR4 on the surface of $CD14^+$ cells were analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Cytokine Production Assays- PBMCs were isolated from 19 C/C subjects, and their controls with the G/C and G/G genotypes. as described above. The cells were resuspended in RPMI 1640 medium (Gibco, Rockville, MD) supplemented with 10% FBS. PBMCs were plated at a density of 1×10^5 cells per well in a 96-well dish and stimulated with 100 ng/ml Pam_3CSK_4 (InvivoGen, San Diego, CA), or 1 ng/ml ultrapure *Escherichia coli* LPS (InvivoGen). Following incubation for 20 h, cell-free supernatants were harvested and analyzed for IL-8, IL-6, and TNF-α production using a commercial ELISA kit (R&D, Minneapolis, MN).

Relative Quantification of TLR4 mRNA levels- PBMCs were isolated from the C/C, G/C, and G/G subjects as described above (n=19 in each genotype group). Total RNA was extracted with RNeasy Mini kit (Qiagen) with on-column DNase treatment according to manufacturers' instructions, and reverse-transcribed for 50 min at 42 °C using 1 U/μl of AMV reverse transcriptase (Promega, Madison, WI) and 60 ng/ml of random primer (Invitrogen, Carlsbad, CA). The cDNA was treated with RNase H (Invitrogen) and purified with QIAprep Spin Miniprepkit (Qiagen). We failed to obtain cDNA from one G/C subject and one G/G subject due to the limitation of blood volumes withdrawn from these subjects; subsequent analysis was performed without these samples. The sequences of the primers and probes for TLR4 were described previously (26). The sequences of the primers and probes for the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were F: 5'-CAT GTT CGT CAT GGG TGT GA-3', R: 5'-CAG GGG TGC TAA GCA GTT GG-3', and Probe: 5'-FAM-CAT CAG CAA TGC CTC CTG CAC CA-TAMRA-3'. These were designed using Primer 3 software and analyzed for homology to other known sequences using the Basic Local Alignment Search Tool (BLAST). In order to avoid the amplification of genomic DNA, and to ensure that the PCR signal was generated from cDNA, primers were placed within different exons

close to intron/exon boundaries. The amplification reactions were performed using Premix Ex Taq (Takara, Shiga, Japan) in a Mx3000 (Stratagene, La Jolla, CA) with a 20 μl of final reaction mixture containing: 2× Premix EX Taq (10 μl); 10 μM (0.4 μl) forward and reverse primers, and probe (final concentration was 0.2 μM); cDNA (2 μl). The thermal profile was as follows: 30 s at 95 °C, and 40 cycles of 5 s at 95 °C and 20 s at 60 °C. Relative quantification of TLR4 mRNA was performed according to the ΔΔCt method (27). THP-1 is a human acute monocytic leukemia cell line that expresses endogenous TLR4. We used this cell line to analyze the effects of miRNAs on TLR4 expression. These cells were stimulated with ultrapure *E. coli* LPS (1 μg/ml), and subjected to the analysis of TLR4 mRNA expression as described above.

miRNA Analysis- miRNA were isolated from PBMCs and THP-1 cells using miRNeasy Kit and RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. qRT-PCR assays were performed using miScript 2 RT Kit, miScript SYBR Green PCR Kit, and miScript Primer Assay. The forward primers for hsa-miR1236, hsa-miR642a, and RNU6B were obtained from Qiagen. The thermal profile was as follows: 15 min at 95 °C, 40 cycles of 15 s at 94 °C, 30 s at 55 °C and 30 s at 70 °C. Relative quantification of hsa-miR1236 and hsa-miR642a was performed according to the ΔΔCt method. An aliquot of these reactions at 38 cycles was electrophoresed on an ethidium bromide-stained 2% agarose gel and specific amplification of the PCR products was confirmed.

Kinetics of TLR4 mRNA, TLR4 protein, and miRNAs- PBMCs were isolated from the G/G and C/C subjects (n=4 in each group) and resuspended in RPMI 1640 medium supplemented with 10% FBS. The cells were plated at a density of 1×10^6 cells per well in a 24-well dish. THP-1 cells in logarithmic growth were resuspended in RPMI 1640 medium supplemented with 10% FBS. The cells were plated at a density of 6×10^5 cells per well in a 24-well dish, and treated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 36 h to induce differentiation into macrophage-like cells. PBMCs and THP-1 cells were stimulated with 100 ng/ml of Pam_3CSK_4 and 1 μg/ml of ultrapure *E. coli* LPS, respectively. Following incubation, a portion of PBMCs and THP-1cells underwent qRT-PCR analysis to determine the relative expression of TLR4 mRNA as described above. Another portion of PBMCs was stained with anti-TLR4 mouse IgG (clone HTA125) and a goat anti-mouse IgG-PE（Beckman Coulter, Fullerton, CA）, washed, resuspended in 2% formaldehyde, washed again, resuspended in the solution consisting of 10% DMSO and 90% FBS, and stored at –80 °C for batch analysis. On the day of analysis, cells from each subject were thawed and surface expression levels of TLR4 of PBMCs were analyzed by flow cytometry (FACSCanto II, Becton Dickinson). Data were analyzed using FlowJo software. For the miRNA analysis, PBMCs from the G/G subjects (n=3) and differentiated THP-1 cells were stimulated with 0.1-1 μg/ml of ultrapure *E. coli* LPS. Following incubation, miRNA were extracted and levels of hsa-miR1236 and hsa-miR642a were quantified as described above.

Plasmid Construction and Luciferase Assays- A 4.3-kbp genomic fragment of the human TLR4 promoter (-4121 to +190) was amplified from human genomic DNA using the Expand High Fidelity PCR system (Roche Biochemicals, Basel,

Switzerland) with primers 5'-CTC CAT GGC ACA TTC TGC AGT AAA CTT GGA GGC-3' (sense) and 5'-CAC GCA GGA GAG GAA GGC CAT GGC TG-3' (antisense). The thermal cycling condition was 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 62 °C, 4 min at 68 °C, and 7 min at 68 °C. The PCR fragment was inserted into the plasmid vector pCR2.1-TOPO (TOPO Cloning Kit, Invitrogen) for subcloning. The 4.3-kb genomic PCR fragment of the TLR4 promoter was subcloned into the *Nco*I restriction site of pGL3-Basic (Promega, Madison, WI) and sequenced (-4121LUC). Deletion of this construct (-742 to +190) was generated by digestion with *Hind*III and subsequent religation of the remaining plasmid (-742LUC). Two types of 219 bp genomic fragments (G/G and C/C) of the 3'-UTR of TLR4 at SNP rs11536889 were amplified from two different human genomic DNAs with the Expand High Fidelity PCR system using the primer sets [5'-TGG GAT CCC TCC CCT GTA CCC TTC-3' (sense) and 5'-CTG GAT CCG TTT CTG AGG AGG CTG GAT G-3' (antisense)]. They were digested with *Bam*HI and inserted into the *Bam*HI restriction site of -4121LUC (-4121LUC+3'GG and -4121LUC+3'CC) and -742LUC (-742LUC+3'GG and -742LUC+3'CC). All constructs were sequenced to verify fidelity. Exponentially growing THP-1 cells were used for transfection assays. The cells were resuspended in Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), and penicillin and streptomycin (Invitrogen). Twenty-four h after plating, the cells at 40~60 % confluence were transfected using a lipofectamin reagent (Invitrogen). The transfection mixture included 1 μg of the luciferase (LUC) construct and 2 μg pSV-β-galactosidase control vector (Promega) as an internal control. In the miRNA inhibition assay, 375 ng of anti-hsa-miR1236, anti-hsa-miR642a, or AllStars Negative Controls (Qiagen) was co-transfected. In the miRNA transfection assay, mutant hsa-miR1236 mimics (mut-miR-1236) and mutant hsa-miR642a mimics (mut-miR-642a) that were predicted to interact with rs11536889 C allele were synthesized (Fig. 9A). The mutant miRNA mimics (37.5 ng), or AllStars Negative Controls (375 ng) was co-transfected. Two days post-transfection, the cells were deprived of serum for 12 hours, and 1 μg/ml of *E. coli* LPS (L2630, *E. coli* O111:B4, Sigma-Aldrich) or 1 ng/ml of human recombinant IL-6 (Wako Pure Chemical Industries, Tokyo, Japan) was added 3 h prior to harvesting. The luciferase activity was measured according to the supplier's protocol using a luminescence reader Accuflex Lumi 400 .

miRNA Inhibition Assay- THP-1 cells were plated at a density of 8×10^5 cells per well in a 24-well dish and treated with 100 ng/ml of PMA for 24 h. The cells were transfected with 375 ng of anti-hsa-miR1236, anti-hsa-miR642a, AllStars Hs Cell Death Control siRNA (Qiagen), or AllStars Negative Controls using a HiPerFect Transfection reagent (Qiagen) according to the manufacturer's protocol. Twelve h post-transfection, the cells were stimulated with 1 μg/ml of ultrapure *E. coli* LPS. Following 24 h incubation, the cells were analyzed for expression of cell surface TLR4 as described in '*Kinetics of TLR4 mRNA, TLR4 protein and miRNAs*'.

Statistical Calculation- Comparisons between the two groups were performed by the Mann-Whitney test unless otherwise indicated. For the luciferase assay, comparisons between the two groups were performed by the unpaired Student's *t*-test. Comparisons between multiple

groups were performed by the Kruskal-Wallis H-test with the Newman-Keuls-test.

RESULTS

PBMCs were isolated from subjects with the G/G, G/C, or C/C genotype of rs11536889, and stained with PE-labeled anti-CD14 and FITC-labeled anti-TLR2, FITC-labeled anti-TLR4, or FITC-labeled isotype-matched control monoclonal antibody. Monocytes were identified based on their CD14 positivity and TLR2 or TLR4 expression was analyzed by flow cytometry. Representative results of the flow cytometric analyses of each genotype are shown in Fig. 1A. Monocytes from C/C subjects expressed significantly higher levels of TLR4 ($p < 0.01$) than those from G/G and G/C subjects, whereas no significant difference was found in the staining with anti-TLR2 or control antibodies (Fig. 1B). When PBMCs were stimulated with LPS, a TLR4 ligand, the cells from C/C and G/C subjects secreted significantly higher levels of IL-8 than the cells from G/G subjects (Fig. 2A). PBMCs from C/C subjects secreted more than twofold higher levels of IL-6 and TNF-α than the cells from the subjects with the other genotypes, but this was not statistically significant (Figs. 2B & 2C). These results suggested that the level of TLR4 protein expression was associated with the response to LPS. When PBMCs were stimulated with Pam_3CSK_4, a TLR2 ligand, no significant difference was found in IL-8, IL-6, and TNF-α levels produced by cells from C/C, G/C, and G/G subjects. To determine whether these differences in TLR4 protein expression was due to the regulation of transcriptional activity by rs11536889 polymorphism, mRNA expression for TLR4 in PBMCs from subjects with each genotype were examined. However, there was no significant difference in the mRNA expression for TLR4 (Fig. 3). Then, we stimulated PBMCs from the G/G and C/C subjects with Pam_3CSK_4 and compared the kinetics of mRNA and protein expression for TLR4 (Fig. 4A). TLR4 mRNA expression in PBMCs from the G/G and C/C subjects increased 2.5 ± 0.9- and 2.1 ± 0.6-fold, respectively, 2 h following the stimulation. Both decreased nearly to the base line levels 3 h following stimulation. There was no significant difference between TLR4 mRNA expression in PBMCs from the G/G subjects and that from the C/C subjects. TLR4 protein expression of PBMCs from the C/C subjects increased 2.2 ± 1.6-, 1.7 ± 0.5- and 1.5 ± 0.3-fold at 10, 20, and 30 h following stimulation, respectively. In contrast, the levels of TLR4 protein on PBMCs from the G/G subjects did not increase by 30 h following stimulation. Fold induction of TLR4 protein on PBMC from the C/C subjects were significantly greater than those on PBMCs from the G/G subjects at 10, 20, and 30 h after stimulation (Fig. 4B).

To determine the effects of rs11536889 polymorphism on the gene expression, transient transfection analyses in THP-1 cells were performed using a series of chimeric constructs containing the human TLR4 promoter ligated to a luciferase reporter and TLR4 3'-UTR. Luciferase activity of pGL3-Basic construct was increased approximately 1.6-fold by the insertion of TLR4 promoter -4121 to +190 bp (-4121LUC) or -742 to +190 bp (-742LUC) (Fig. 5). Luciferase activity of the TLR4 promoter construct was further increased by stimulation with 1 μg/ml of LPS or 1 ng/ml of IL-6. The insertion of 219 bp fragment of 3'-UTR containing rs11536889 G allele (-4121LUC+3'GG and

-742LUC+3'GG) inhibited this up-regulation. The insertion of a 219 bp fragment of 3'-UTR containing the rs11536889 C allele (-4121LUC+3'CC and -742LUC+3'CC) did not inhibit the up-regulation.

To identify a possible causal effect of the G to C nucleotide transversion of SNP rs11536889, we analyzed the secondary structure of the mRNA (Vienna RNA Package 1.8.4.; http://rna.tbi.univie.ac.at). The exchange had no effect on the mRNA folding; the minimum free energy of the optimal secondary structure of G and C allele were -1813.12 and -1813.86 kcal/mol, respectively, making a marginal difference. A prediction for miRNA targets (microRNA resource; http://www.microrna.org) identified hsa-miR-1236 and hsa-miR-642a as candidates for miRNAs that bind to the region containing rs11536889 G allele (Fig. 6A). The altered nucleotide is located within the potential binding sites of hsa-miR-1236 and hsa-miR-642a and introduces a mismatch at the 6th and 13th positions, respectively. Both hsa-miR-1236 and hsa-miR-642a were expressed in THP-1 cells (Fig. 6B). The level of hsa-miR-1236 was increased 10 h following LPS stimulation, whereas that of TLR4 mRNA was increased 2 h, but decreased nearly to the base line levels 3 h following LPS stimulation. No up-regulation of hsa-miR-642a was induced by LPS stimulation (Fig. 6C). hsa-miR-1236 and hsa-miR-642a were also detected in PBMCs, however, the kinetics following LPS stimulation varied widely between the individuals (Fig. 6D). When luciferase constructs were co-transfected with the inhibitors for hsa-miR-642a and/or hsa-miR-1236, the repression of luciferase activity of -4121LUC+3'GG was reversed (Figs. 7A & 7B). Importantly, the inhibitors for the miRNAs did not affect the luciferase activities of -4121LUC+3'CC, indicating that the effect of these inhibitors was specific for the rs11536889 G allele.

Since THP-1 cells express endogenous TLR4, we used these cells to examine the effects of miR-642a and miR-1236 on TLR4 expression. The cells were genotyped for rs11536889 by PCR-CTPP and were shown to be G/G (data not shown). There was no mismatch between the TLR4 sequence of THP-1 cells and that of transient transfection constructs of the G allele. These cells were transfected with the miRNA inhibitors or control siRNA. In each transfection, transfection efficiency was confirmed using AllStars Hs Cell Death Control siRNA. When the cells were stimulated with LPS, the levels of TLR4 of the cells transfected with anti-miR-642a, anti-miR-1236, and the combination of both were statistically higher than that of the control indicating that both miR-642a and miR-1236 contributed to the suppression of TLR4 on THP-1 cells (Fig. 8B). When the cells were left unstimulated, TLR4 expression on the cells transfected with anti-miR-642a and/or anti-miR-1236 was slightly increased, but no statistical difference was found (Fig. 8A).

As hsa-miR-1236 and hsa-miR-642a were predicted to interact only with the rs11536889 G allele, we synthesized mutant miRNAs, mut-miR-1236 and mut-miR-642a, that were predicted to interact only with the rs11536889 C allele (Fig. 9A). When luciferase constructs were transfected simultaneously with mut-miR-642a, mut-miR-1236, or the combination of both, the luciferase activity of -4121LUC+3'CC was inhibited (Figs. 9B & 9C). Importantly, the inhibitors for the miRNAs did not change the luciferase activity of -4121LUC+3'GG, indicating that the effect of inhibitors was specific for the

rs11536889 C allele.

DISCUSSION

The peripheral blood monocytes from the C/C subjects expressed significantly higher levels of TLR4 protein on their surfaces than those from the G/G and G/C subjects in the present study. When PBMCs were stimulated with Pam_3CSK_4, the levels of TLR4 protein on their surface significantly increased in the C/C subjects, whereas no increase was detected in the G/G subjects. This suggested that TLR4 protein expression was down-regulated in the G/G subjects or up-regulated in the C/C subjects. Induction of luciferase activity in THP-1 cells transfected with a TLR4 promoter construct was inhibited by insertion of a 219 bp fragment of 3'-UTR containing the rs11536889 G allele, but not the C allele. Considering that no other mismatches were found in the nucleotide sequences between these two 219 bp fragments (data not shown), TLR4 protein expression seems to be down-regulated by a small region containing the rs11536889 G allele in 3'-UTR. One study demonstrated that the expression of TLR4 on human monocytes was not affected by LPS stimulation (28) but another study showed that LPS up-regulated the expression of TLR4 on human monocytes (29). The distinct behaviors of TLR4 in these studies might be due to differences in the donor genotype.

In contrast to the clear difference between TLR4 protein expression on the monocytes from the G/G, G/C, and C/C subjects, there was no significant difference in TLR4 mRNA expression levels in PBMCs in the present study. TLR4 mRNA levels in PBMCs from both the G/G and C/C subjects were elevated 2 h following stimulation with Pam_3CSK_4. Howeve, the TLR4 protein levels on PBMCs increased only in the C/C subjects, but not in the G/G subjects. This evidence suggested that the genetic variation at rs11536889 affected translation rather than transcription of TLR4.

Anti-miR-642a and anti-miR-1236 up-regulated TLR4 expression on the surface of THP-1 cells stimulated with LPS. This result suggests that TLR4 expression of the subjects was, at least in part, inhibited by miR-642a and miR-1236. These miRNA inhibitors also up-regulated the luciferase activity of -4121LUC+3'GG, but did not affect the luciferase activity of -4121LUC+3'CC. Conversely, mut-miR-642a and mut-miR-1236 suppressed the luciferase activity of -4121LUC+3'CC, but did not affect the luciferase activity of -4121LUC+3'GG. These results strongly suggest that binding of the miRNAs is specific to each genotype. miRNAs usually base-pair to target mRNA with imperfect complementation, resulting in translational inhibition, whereas perfect base-pairing induces target mRNA degradation (30). The imperfect complementation between miR-642a and TLR4 mRNA (mirSVR score (31): -0.3230) and between miR-1236 and TLR4 mRNA (mirSVR score: -0.2059) may lead to translational inhibition. Although both miR-642a and miR-1236 were expressed in PBMCs and THP-1 cells, the expression of these miRNAs may be different in other cell types (32,33). The inhibitory effects of these miRNAs on TLR4 expression in different tissues remain to be elucidated.

PBMCs from the C/C and G/C subjects secreted significantly higher levels of IL-8 in response to LPS than PBMCs from the G/G subjects. This could be due to the higher expression of TLR4 in the C/C subjects. In

accordance, Bihl et al. showed that increased expression of TLR4 resulted in a more sensitive response to LPS (34). PBMCs from the C/C subjects secreted slightly higher levels of IL-6 and TNF-α than cells from the subjects with the other genotypes; however, there was no significant difference, even when the number of subjects in each group was increased from 12 to 19. It is known that MD-2 as well as CD14 are essential for the recognition of LPS (35). In addition, the intracellular signaling events following LPS-binding to TLR4 could affect the magnitude of cytokine responses (5). The inter-individual variation of these molecules could affect sensitivity to LPS and may result in the ambiguous differences in IL-6 and TNF-α levels. Although PBMCs from the G/C subjects secreted higher levels of IL-8 than those from G/G subjects, no significant difference was seen in TLR4 expression on the surface of peripheral blood monocytes. The causal relationship between G/C genotype and TLR4 expression and function needs to be investigated further.

In our previous study, the genetic variation of rs11536889 was associated with periodontitis. The C/C genotype was observed more frequently in the periodontitis group than in the control group. The up-regulation of TLR4 expression in the C/C subjects may prompt the recognition of Gram-negative bacterial LPS in periodontal pockets, accelerate the production of pro-inflammatory cytokines, and lead to the greater destruction of periodontal tissue. Kornman *et al*. found that a variant in *IL-1B* associated with high levels of IL-1β production is a strong indicator of increased susceptibility to severe periodontitis (36); this is yet another example how the genetic background prone to accelerated inflammation is linked to periodontitis. The genetic variation of rs11536889 is also associated with severe gastric atrophy in *H. pylori* seropositive Japanese (18), HBV recurrence after liver transplantation (19), and increased prostate cancer risk (19). The associations may be related to accelerated inflammation caused by LPS from *H. pylori* in gastric mucosa, HBV related proteins in the liver, Gram-negative bacteria in the prostate, respectively. A prospective study consisting of the G/G and C/C subjects to reveal a more precise predispositional effect of this SNP on these diseases remains to be performed.

To the best of our knowledge, this is the first report to reveal the biological significance of the genetic variation of rs11536889. This SNP regulates the expression of TLR4 and has some influence on the response to LPS. These findings may provide a novel approach for therapeutic interventions against diseases caused by harmful TLR4-mediated responses.

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FOOTNOTES

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The abbreviations used are: TLR, Toll-like receptor; LUC, luciferase; miRNA, MicroRNA; MFI, mean fluorescence intensity; qRT-PCR, quantitative reverse transcription PCR.

FIGURE LEGENDS

Fig. 1. Expression of TLR4 on the surface of monocytes from subjects classified by rs11536889 genotype. PBMCs from the G/G, G/C, and C/C subjects were stained with PE-labeled anti-CD14 and FITC-labeled anti-TLR2, FITC-labeled anti-TLR4, or FITC-labeled isotype-matched control monoclonal antibodies. $CD14^+$ cells were gated and TLR4 or TLR2 expression was analyzed by flow cytometry. Representative results of the cells from the subjects with each genotype are shown (A). The mean fluorescence intensities are expressed with standard deviations (B). *, $p < 0.05$.

Fig. 2. Cytokine production by PBMCs from subjects classified by rs11536889 genotype. PBMCs from the G/G, G/C, and C/C subjects were stimulated with 1 ng/ml of LPS, 100 ng/ml of Pam_3CSK_4, or left unstimulated. The levels of IL-8 (A), IL-6 (B), or TNF-α (C) are expressed with standard deviations. *, $p < 0.05$; **, $p < 0.01$.

Fig. 3. Expression of TLR4 mRNA in PBMCs from subjects classified by rs11536889 genotype. PBMC were isolated from the G/G, G/C, and C/C subjects, and total RNA was extracted. Following reverse transcription, mRNA levels for TLR4 were determined by qRT-PCR using a specific TaqMan probe. The relative mRNA levels for TLR4 are expressed with standard deviations.

Fig. 4. Kinetics of TLR4 mRNA and protein expression in PBMCs stimulated with Pam_3CSK_4. PBMCs from the G/G and C/C subjects were stimulated with 100 ng/ml of Pam_3CSK_4. Following the indicated time of incubation, a portion of cells underwent qRT-PCR analysis to determine the relative expression of TLR4 mRNA (A). Another portion of cells was stained with anti-TLR4 mouse IgG and a goat anti-mouse IgG-PE and surface expression levels of TLR4 was analyzed by flow cytometry (B). Fold induction is expressed with standard deviations. MFI, mean fluorescence intensity; *, $p < 0.05$ (G/G versus C/C).

Fig. 5. rs11536889 SNP regulates luciferase activity driven by TLR4 promoter in THP-1 cells. A series of chimeric luciferase constructs were analyzed for its expression after transfection into THP-1 cells. The transfectants were left unstimulated, or stimulated with 1 μg/ml of LPS (A) or 1 ng/ml of IL-6 (B) for 3 h; a luciferase activity was examined. The results of the assay obtained from four separate transfections with constructs pGL3-Basic, -4121LUC, -4121LUC+3'GG, -4121LUC+3'CC, -742LUC, -742LUC+3'GG, and -742LUC+3'CC have been combined. The values are expressed with standard errors. *, $p < 0.05$; **, $p < 0.01$ (versus the unstimulated controls).

Fig. 6. Expression of TLR4 mRNA, hsa-miR-1236, and hsa-miR-642a. Sequence alignments of 3'-UTR of human *TLR4* (rs11536889 G allele), hsa-miR-1236 and hsa-miR-642a are shown. The position of SNP rs11536889 is underlined (A). Differentiated THP-1 cells were stimulated with 1 μg/ml of LPS. Following the indicated time of incubation, the cells were harvested; total RNA and miRNA were extracted. cDNA synthesis and amplification was performed as described in experimental procedures. An aliquot of these reactions was electrophoresed on an ethidium bromide-stained 2% agarose gel (B). Levels of TLR4 mRNA, hsa-miR-1236, and hsa-miR-642a were measured by qRT-PCR (C). Fold induction is expressed with standard deviations. *, $p < 0.05$ (versus the unstimulated controls). Freshly isolated PBMCs were stimulated with 100 ng/ml of LPS. Following the indicated time of incubation, the cells were harvested and analyzed for hsa-miR-1236 or hsa-miR-642a expression levels as described in experimental procedures (D). Fold induction is expressed with standard deviations. *, $p < 0.05$ (versus the unstimulated controls).

Fig. 7. Effects of microRNA inhibitors on luciferase constructs in THP-1 cells. THP-1 cells were transfected with a series of chimeric luciferase constructs in the presence or absence of miRNA inhibitors. The transfectants were left unstimulated or stimulated with 1 μg/ml of LPS (A) or 1 ng/ml of IL-6 (B) for 3 h; luciferase activity was examined. The results of the assay obtained from three separate transfections

with constructs pGL3-Basic, -4121LUC, -4121LUC+3'GG, and -4121LUC+3'CC with or without miRNA inhibitor have been combined. The values are expressed with standard errors. *, $p < 0.05$; **, $p < 0.01$ (versus the unstimulated controls).

Fig. 8. Effects of microRNA inhibitors on TLR4 expression on the surface of THP-1 cells. THP-1 cells were transiently transfected with miRNA inhibitors against miR1236 and/or miR642a. Following 12 h, the cells were left unstimulated (A) or stimulated with 100 ng/ml of LPS (B), incubated for 24 h. Surface TLR4 expression was analyzed as described in experimental procedures. The mean fluorescence intensities (MFIs) were expressed with standard deviations. Representative results are shown. *, $p < 0.05$ (versus the negative controls).

Fig. 9. Inhibitory effects of mutant miRNAs that bind to 3'-UTR of *TLR4* with the rs11536889 C allele. Sequence alignments of 3'-UTR of human *TLR4* (rs11536889 C allele), mutant hsa-miR-1236 (mut-miR-1236), and mutant hsa-miR-642a (mut-miR-642a) are shown (A). The position of SNP rs11536889 is underlined. THP-1 cells were transfected with a series of chimeric luciferase constructs in the presence or absence of mutant miRNAs. The transfectants were stimulated with 1 μg/ml of LPS (B) or 1 ng/ml of IL-6 (C) for 3 h; luciferase activity was examined. The results of the assay are obtained from four separate transfections with constructs pGL3-Basic, -4121LUC, -4121LUC+3'GG, and -4121LUC+3'CC with or without mutant miRNAs have been combined. The values are expressed with standard errors. *, $p < 0.05$ (versus the unstimulated controls).

Table 1. Basic Characteristics of the Study Population.

		G/G	G/C	C/C
Flow cytometry	M/F	7/5	6/6	6/6
	Age	58.6 ± 13.3	62.5 ± 11.9	59.5 ± 12.7
ELISA	M/F	11/8	10/9	10/9
	Age	58.1 ± 13.7	59.9 ± 13.8	58.7 ± 13.8
qRT-PCR	M/F	11/7	9/9	10/9
	Age	57.7 ± 14.0	58.8 ± 13.4	58.7 ± 13.8
Time course	M/F	3/1		3/1
	Age	54.0 ± 16.6		57.3 ± 20.8

M: Male, F: Female

Age is expressed with standard deviations.

Fig. 1

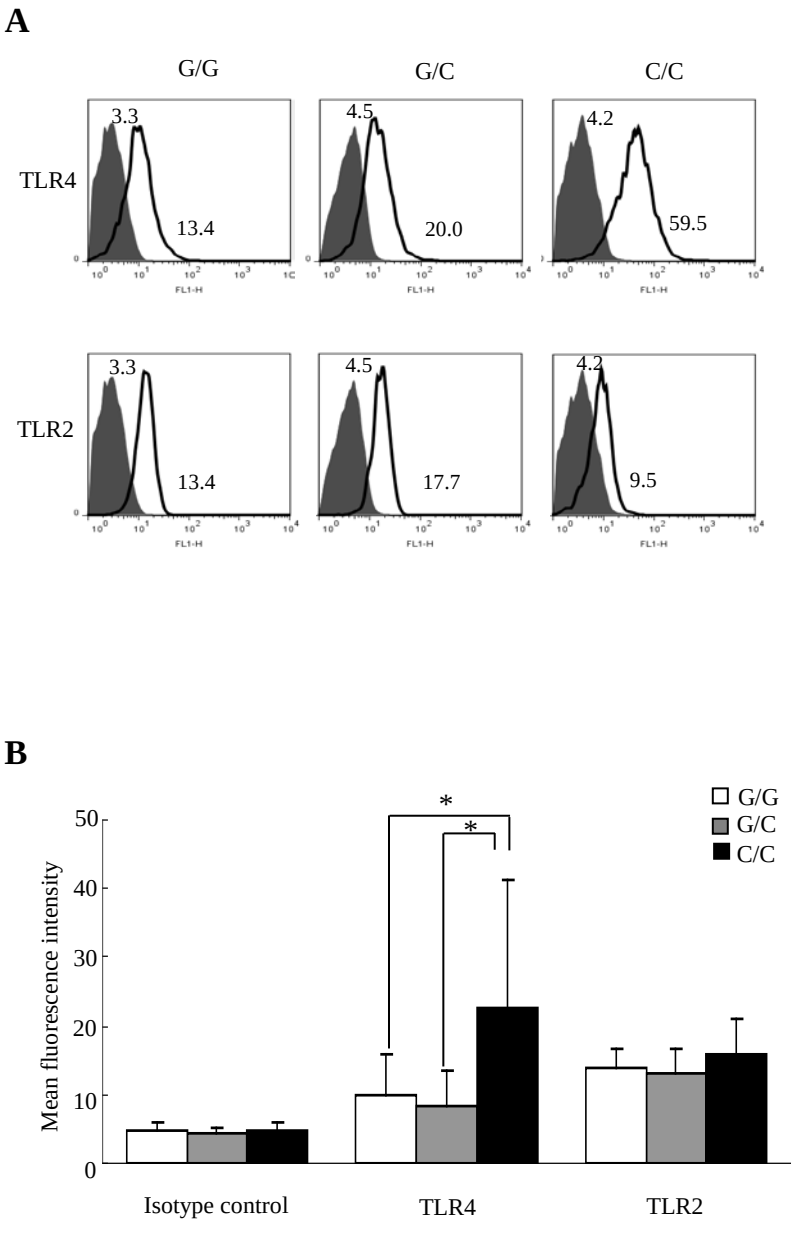

A
G/G
G/C
C/C
TLR4
3.3
13.4
4.5
20.0
4.2
59.5
TLR2
3.3
13.4
4.5
17.7
4.2
9.5
FL1-H
B
G/G
G/C
C/C
Mean fluorescence intensity
50
40
30
20
10
0
*
*
Isotype control
TLR4
TLR2

Fig. 2

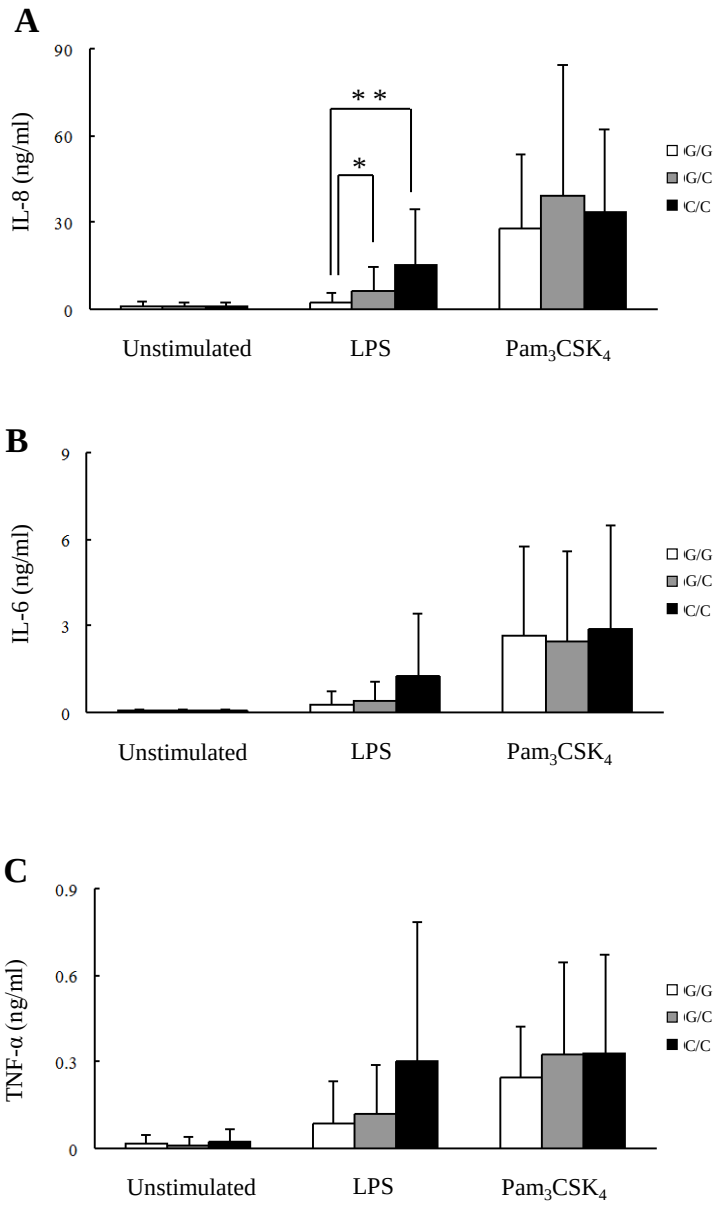

A
IL-8 (ng/ml)
90
60
30
0
* *
*
G/G
G/C
C/C
Unstimulated
LPS
Pam3CSK4
B
IL-6 (ng/ml)
9
6
3
0
G/G
G/C
C/C
Unstimulated
LPS
Pam3CSK4
C
TNF-α (ng/ml)
0.9
0.6
0.3
0
G/G
G/C
C/C
Unstimulated
LPS
Pam3CSK4

Fig. 3

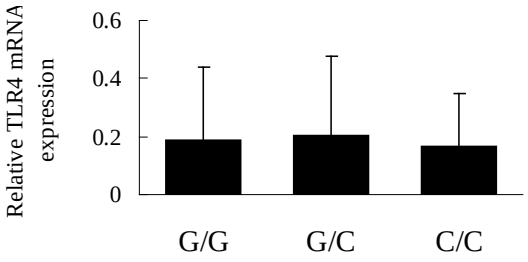
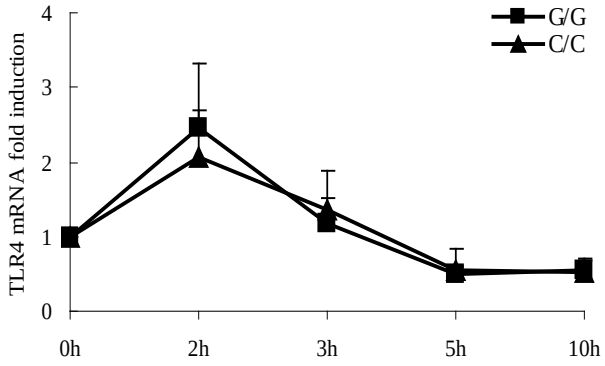


Fig. 4

A


TLR4 mRNA fold induction
4
3
2
1
0
0h
2h
3h
5h
10h
G/G
C/C

B

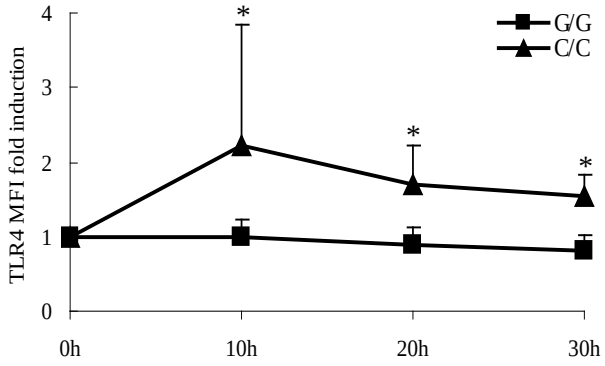

TLR4 MFI fold induction
4
3
2
1
0
0h
10h
20h
30h
*
*
*
G/G
C/C

Fig. 5

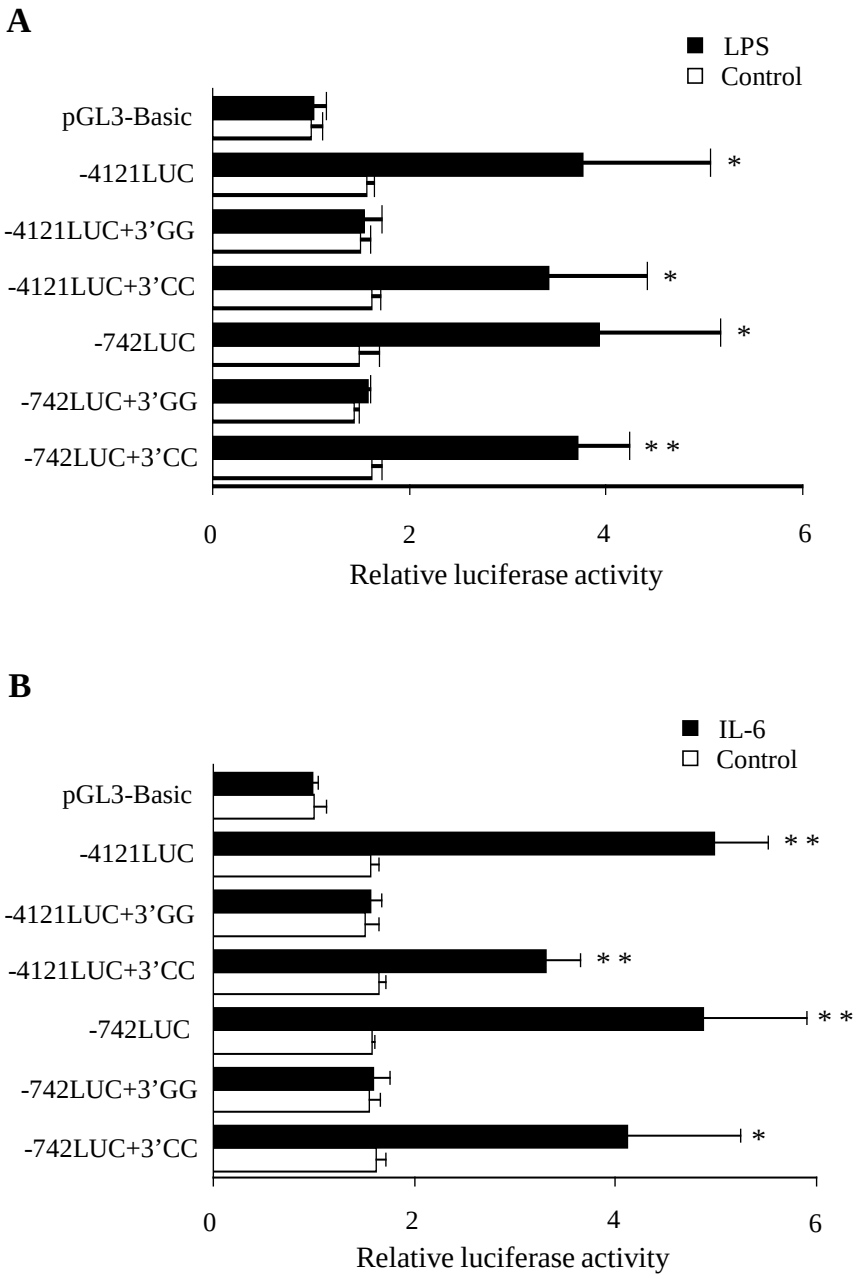

A
LPS
Control
pGL3-Basic
-4121LUC
-4121LUC+3'GG
-4121LUC+3'CC
-742LUC
-742LUC+3'GG
-742LUC+3'CC
*
*
*
* *
0
2
4
6
Relative luciferase activity
B
IL-6
Control
pGL3-Basic
-4121LUC
-4121LUC+3'GG
-4121LUC+3'CC
-742LUC
-742LUC+3'GG
-742LUC+3'CC
* *
* *
* *
*
0
2
4
6
Relative luciferase activity

Fig. 6

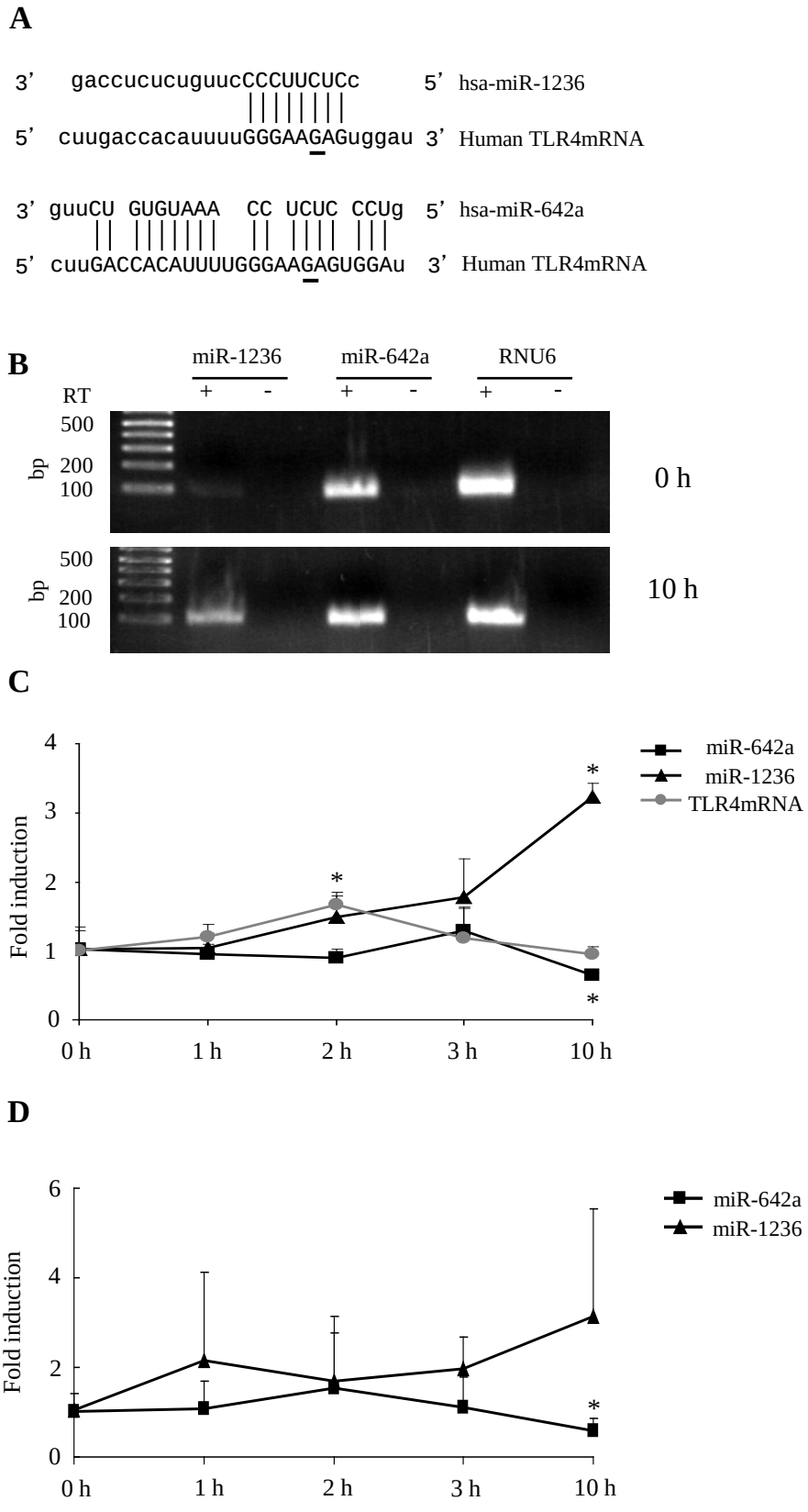

A
3’ gaccucucuguucCCCUUCUCc 5’ hsa-miR-1236
5’ cuugaccacauuuuGGGAAGAGuggau 3’ Human TLR4mRNA
3’ guuCU GUGUAAA CC UCUC CCUg 5’ hsa-miR-642a
5’ cuuGACCACAUUUUGGGAAGAGUGGAu 3’ Human TLR4mRNA
B
miR-1236
miR-642a
RNU6
RT
+ - + - + -
500
200
100
bp
0 h
10 h
C
Fold induction
miR-642a
miR-1236
TLR4mRNA
0 h
1 h
2 h
3 h
10 h
D
Fold induction
miR-642a
miR-1236
0 h
1 h
2 h
3 h
10 h

Fig. 7

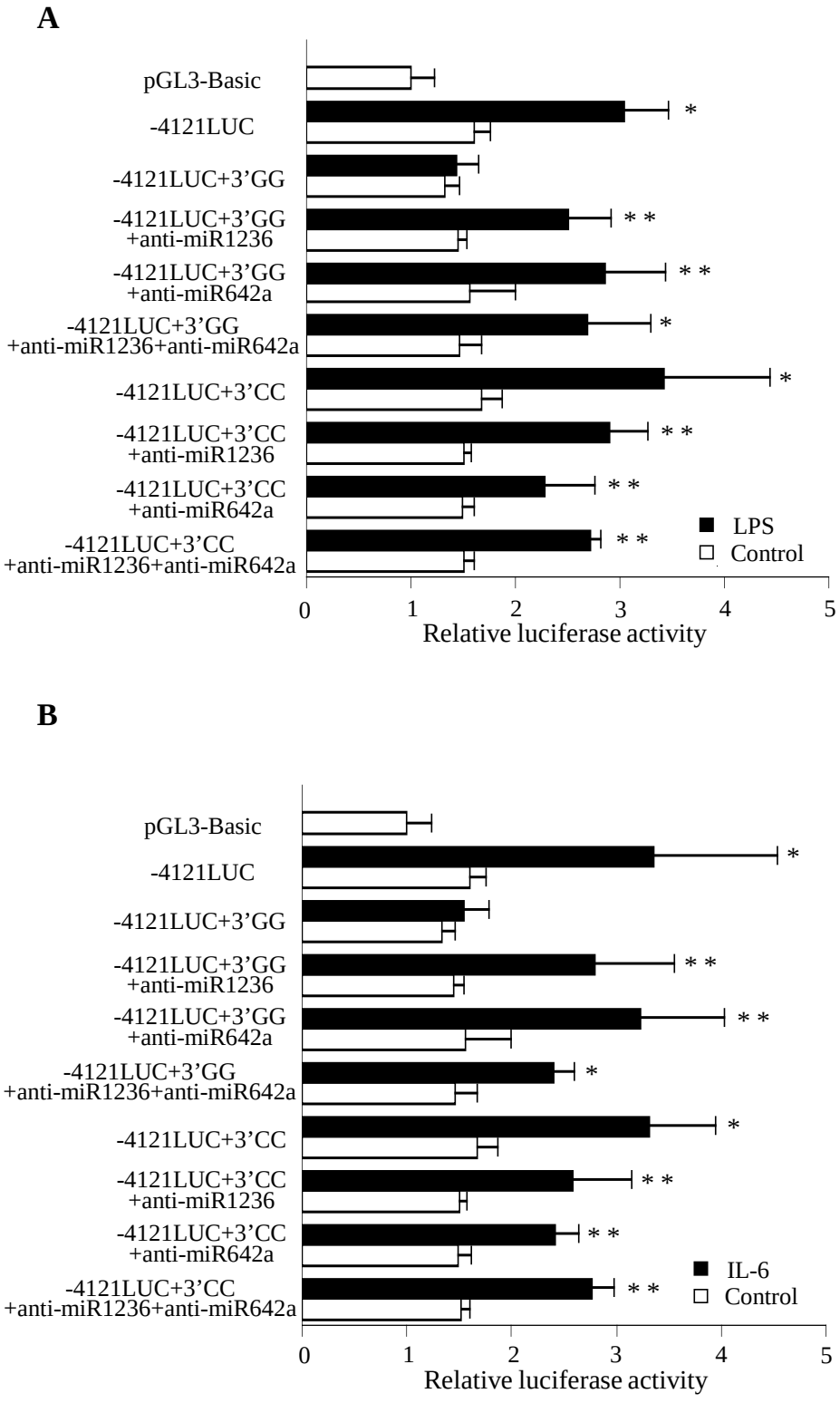

A
pGL3-Basic
-4121LUC
-4121LUC+3'GG
-4121LUC+3'GG +anti-miR1236
-4121LUC+3'GG +anti-miR642a
-4121LUC+3'GG +anti-miR1236+anti-miR642a
-4121LUC+3'CC
-4121LUC+3'CC +anti-miR1236
-4121LUC+3'CC +anti-miR642a
-4121LUC+3'CC +anti-miR1236+anti-miR642a
LPS
Control
0 1 2 3 4 5
Relative luciferase activity
B
pGL3-Basic
-4121LUC
-4121LUC+3'GG
-4121LUC+3'GG +anti-miR1236
-4121LUC+3'GG +anti-miR642a
-4121LUC+3'GG +anti-miR1236+anti-miR642a
-4121LUC+3'CC
-4121LUC+3'CC +anti-miR1236
-4121LUC+3'CC +anti-miR642a
-4121LUC+3'CC +anti-miR1236+anti-miR642a
IL-6
Control
0 1 2 3 4 5
Relative luciferase activity

Fig. 8

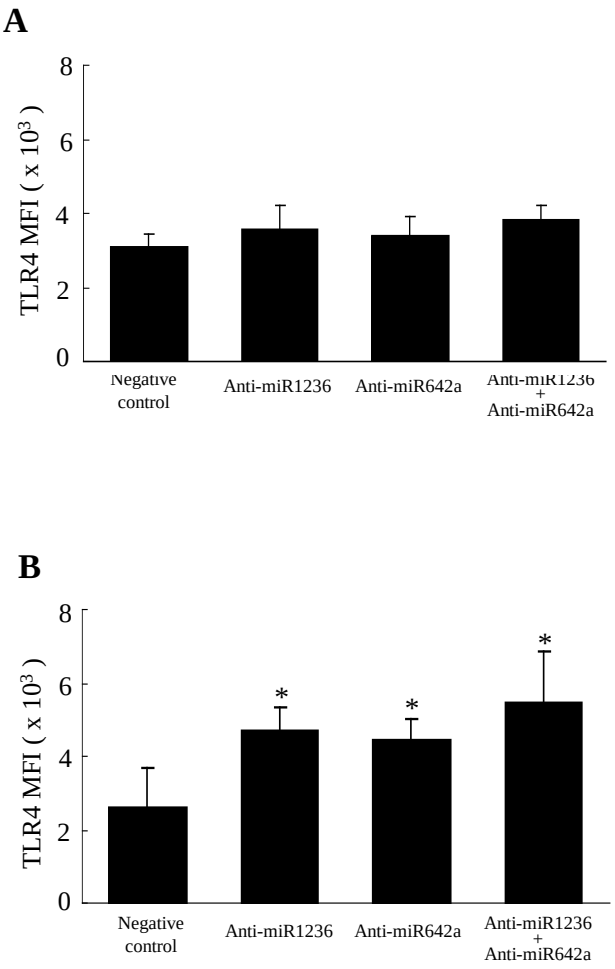
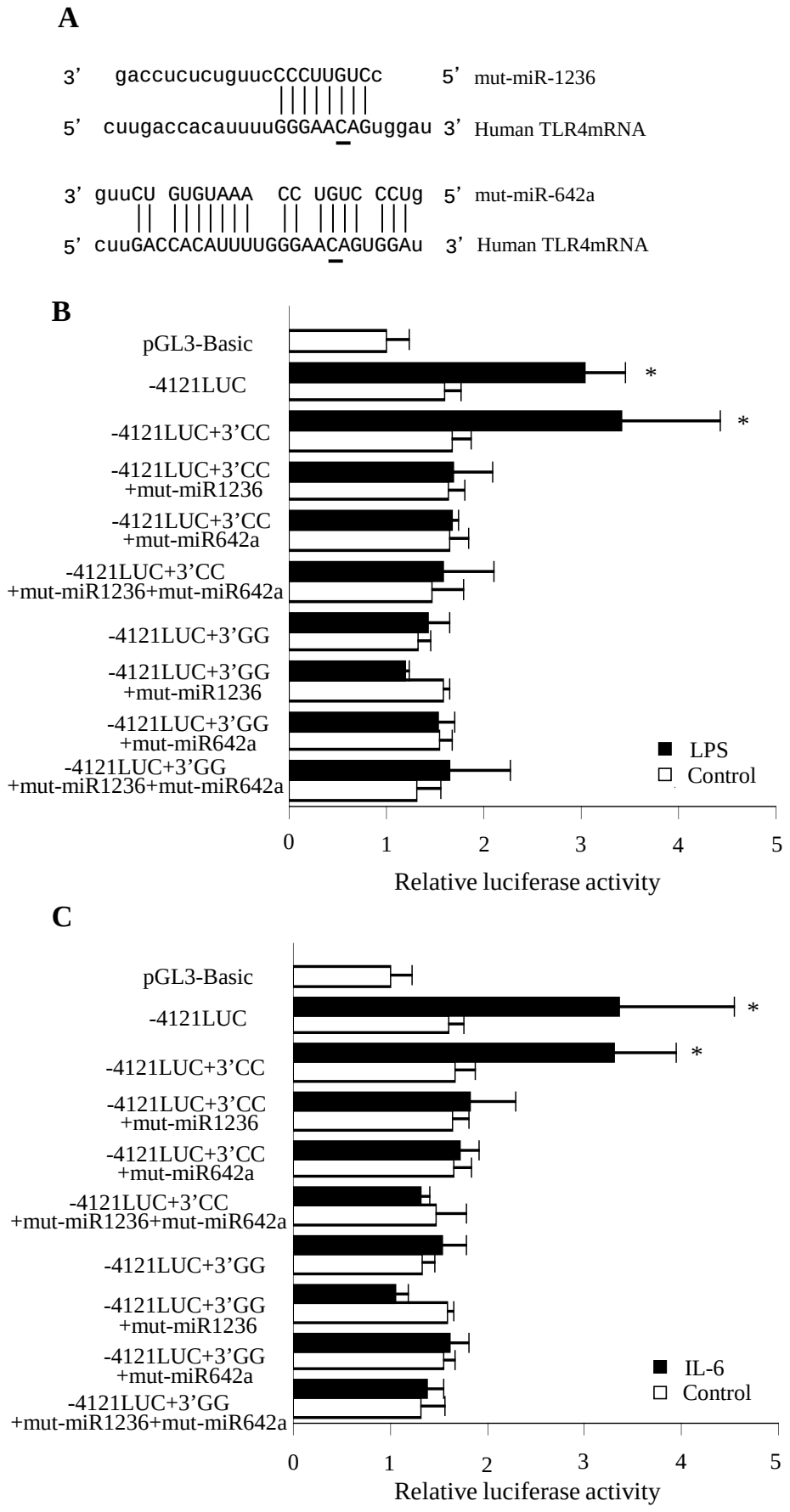

A
TLR4 MFI (x 10³)
0
2
4
6
8
Negative control
Anti-miR1236
Anti-miR642a
Anti-miR1236 + Anti-miR642a
B
TLR4 MFI (x 10³)
0
2
4
6
8
*
*
*
Negative control
Anti-miR1236
Anti-miR642a
Anti-miR1236 + Anti-miR642a

Fig. 9


A
3’ gaccucucuguucCCCUUGUCc 5’ mut-miR-1236
5’ cuugaccacauuuuGGGAACAGuggau 3’ Human TLR4mRNA
3’ guuCU GUGUAAA CC UGUC CCUg 5’ mut-miR-642a
5’ cuuGACCACAUUUUGGGAACAGUGGAu 3’ Human TLR4mRNA
B
pGL3-Basic
-4121LUC
-4121LUC+3’CC
-4121LUC+3’CC +mut-miR1236
-4121LUC+3’CC +mut-miR642a
-4121LUC+3’CC +mut-miR1236+mut-miR642a
-4121LUC+3’GG
-4121LUC+3’GG +mut-miR1236
-4121LUC+3’GG +mut-miR642a
-4121LUC+3’GG +mut-miR1236+mut-miR642a
LPS
Control
0 1 2 3 4 5
Relative luciferase activity
C
pGL3-Basic
-4121LUC
-4121LUC+3’CC
-4121LUC+3’CC +mut-miR1236
-4121LUC+3’CC +mut-miR642a
-4121LUC+3’CC +mut-miR1236+mut-miR642a
-4121LUC+3’GG
-4121LUC+3’GG +mut-miR1236
-4121LUC+3’GG +mut-miR642a
-4121LUC+3’GG +mut-miR1236+mut-miR642a
IL-6
Control
0 1 2 3 4 5
Relative luciferase activity