Calprotectin induces IL-6 and MCP-1 Production via Toll-like Receptor 4 Signaling in Human Gingival Fibroblasts

Yasufumi Nishikawa, Yukari Kajiura, Jung Hwan Lew, Jun-ichi Kido, Toshihiko Nagata, Koji Naruishi*

Department of Periodontology and Endodontology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto, Tokushima 770-8504, Japan

***Corresponding author:** Dr. Koji Naruishi, D.D.S., Ph.D.

Department of Periodontology and Endodontology, Institute of Health Biosciences, Tokushima University Graduate School, 3-18-15 Kuramoto, Tokushima 770-8504, Japan

Phone: +81-88-631-3111; Fax: +81-88-633-7009

e-mail: naruishi@tokushima-u.ac.jp

Running title: Calprotectin/TLR4 induced-Cytokine Production

Keywords: calprotectin, IL-6, MCP-1, TLR4, gingival fibroblasts

Total number of text figures: 8 figures, tables: 0 table.

Contract grant sponsor: the Japan Society for the Promotion of Science

Contract grant number: Grant-in-Aid for Scientific Research (B) (No. 15H05054), Scientific Research (C) (No. 16K11832) and Grant-in-Aid for Young Scientists (B) (No. 15K20621) from the Japan Society for the Promotion of Science.

Abstract

Calprotectin, a heterodimer of S100A8 and S100A9 molecules, is associated with inflammatory diseases such as inflammatory bowel disease. We have reported that calprotectin levels in gingival crevicular fluids of periodontitis patients are significantly higher than in healthy subjects. However, the functions of calprotectin in pathophysiology of periodontitis are still unknown. The aim of this study is to investigate the effects of calprotectin on the productivity of inflammatory cytokines in human gingival fibroblasts (HGFs). The HGFs cell line CRL-2014® (ATCC) were cultured, and total RNAs were collected to examine the expression of TLR2/4 and RAGE mRNA using RT-PCR. After the cells were treated with S100A8, S100A9 and calprotectin, supernatants were collected and the levels of IL-6 and MCP-1 were measured using ELISA methods. To examine the intracellular signals involved in calprotectin-induced cytokine production, several chemical inhibitors were used. Furthermore, after the siRNA-mediated TLR4 down-regulated cells were treated with S100A8, S100A9 and calprotectin, the levels of IL-6 and MCP-1 were also measured. HGFs showed greater expression of TLR4 mRNA, but not TLR2 and RAGE mRNA compared with human oral epithelial cells. Calprotectin increased significantly the production of MCP-1 and IL-6 in HGFs, and the cytokine productions were significantly suppressed in the cells treated with MAPKs, NF-κB and TLR4 inhibitors. Furthermore, calprotectin-mediated MCP-1 and IL-6 production were significantly suppressed in TLR4 down-regulated cells. Taken together, calprotectin induces IL-6 and MCP-1 production in HGFs *via* TLR4 signaling that involves MAPK and NF-κB, resulting in the progression of periodontitis.

Introduction

Periodontitis is a bacterial infectious disease, and many inflammatory cytokines regulate the pathophysiology through the tissue cells and immune cells-interactions (Graves, 2003; Takashiba, 2003). Neutrophils are the most abundant of white blood cells, which play a key role in the innate immune system by producing several cytokines (Bao, 2014). As a key effector cell in early stage of inflammation, increased number of neutrophils are found in inflammatory lesions. S100A8 and S100A9 are expressed primarily in neutrophils, constituting approximately over than 40% of the cytosolic proteins (Cesaro, 2012). S100A8 and S100A9 can form homo- or hetero-complexes, the latter known as calprotectin, both forms are abundantly released from neutrophils under inflammatory conditions such as inflammatory bowel disease (Burri, 2014) and periodontitis (Nisapakultorn, 2001). Elevated calprotectin might indicate the infiltration of neutrophils to the intestinal mucosa or periodontal connective tissues which occurs inflammation. We have reported previously that calprotectin levels in gingival crevicular fluids (GCFs) of inflamed periodontal sites are significantly higher than in healthy sites (Kido, 1999). Calprotectin might be one of attractive molecules for the diagnosis of periodontitis.

There are many reports about calprotectin function in inflammatory diseases. Previously, calprotectin has been reported as an antimicrobial molecule with calcium binding properties (Steinbakk, 1990). On the other hand, it has been reported that calprotectin increased interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF) expression in periodontal ligament cells (Zheng, 2014). Thus, although calprotectin must be a multifactorial molecule in inflammatory lesions such as periodontitis, the effects of

calprotectin on gingival fibroblasts remains unclear.

Human gingival fibroblasts (HGFs) are the most abundant cells in gingival connective tissues and play an important role in the regulation of inflammatory process, and the functions are well controlled by various cytokines (Takashiba, 2003). Our previous studies have shown that IL-1β induces IL-6 or monocyte chemoattractant protein (MCP)-1 production in HGFs (Sawada, 2013). IL-6 and MCP-1 have been implicated in the progression of periodontitis (Okada, 1998). IL-6 enhances osteoclastic activity (Blair, 2004) and collagen destruction (Beklen, 2007) in periodontitis lesion. In addition, IL-6 induces vascular endothelial growth factor (VEGF) and matrix metalloproteases (MMPs) production in the presence of soluble form of IL-6 receptor (sIL-6R) in HGFs (Naruishi, 2003; Sawada, 2013). MCP-1 is one of the most potent chemoattractant for monocytes (Faller, 1997), and elevated MCP-1 has been observed in gingival tissue of periodontitis patients, suggesting its predominant role in increase of monocyte chemotactic activity (Gupta, 2013). Thus, although it is well-known that HGFs are one of important cells regulating cytokine cascades in periodontitis lesion, the biological effects of calprotectin on HGFs function remains unclear. Therefore, we focused on the biological function of calprotectin in HGFs, since calprotectin levels are elevated significantly in periodontitis lesions.

In the present study, we have investigated the effects of calprotectin on HGFs to clarify the cytokine cascades surrounding HGFs. This regulation would be attractive target to clarify the pathophysiology of periodontitis.

Materials and Methods

Reagents

Recombinant human S100A8 and S100A9 were purchased from ATGen Ltd (Sampyeongdong, Korea) and each recombinant proteins were mixed and incubated for 1 h at 4°C to form the calprotectin as described previously (Kwon, 2013). *Porphyromonas gingivalis* (Pg) LPS was purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against phospho- and total p65, IκB, ERK, JNK and p38MAPK were obtained from Cell Signaling Technology (Beverly, MA). Selective NF-κB, ERK, JNK, p38MAPK and androgen receptor inhibitor flutamide were purchased from Selleck (Houston, Tx). MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide] was obtained from Sigma (St. Louis, MO). Selective TLR4 inhibitor TAK242 was purchased from InvivoGen (San Diego, CA). The specific TLR4 inhibitor VIPER and its control peptide (CP7) were purchased from Novus Biologicals (Littleton, CO).

Cell culture

The human oral epithelial cell line isolated from a human buccal carcinoma TR146 was kindly provided by Dr. Herzberg (University of Minnesota, MN, USA). TR146 cells were cultured in Ham's F12 medium supplemented with 10 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (cell growth medium). The human gingival fibroblast cell line CRL-2014® was purchased from ATCC and maintained in DMEM supplemented with 10 % FBS according to the manufacturer's instructions.

Cell Proliferation

To examine the cell viability after stimulation of S100A8, S100A9 and calprotectin, MTT assay was performed as described previously (Yamaguchi, 2008). In short, HGFs were seeded in each well of a 96-well culture plate in a final volume of 100 μl of DMEM medium supplemented with 0.5 % FBS. After cells reached sub-confluent, S100A8, S100A9 and calprotectin (50, 100 nM each) were applied and kept for 24-72 h. At the end of the treatment, MTT (final concentration: 0.5 mg/ml) was added to each well and incubated for 4 h in a humidified atmosphere prior to the addition of 100 μl of DMSO into each well. The reaction mixture on each well of the 96-well culture plate was measured fluorometrically using an auto plate reader (Bio-Rad, Hercules, CA; excitation at 595 nm).

TLR 2/ 4 and RAGE mRNA expression

For RNA analyses by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), oral epithelial cells and HGFs were cultured until sub-confluent. In addition, after HGFs reached sub-confluent, calprotectin (50 nM) was applied and kept for 24-48 h. Total RNA was isolated from cells using RNeasy® Mini Kit (QIAGEN, Hilden, Germany), and first-strand cDNA was synthesized with PrimeScript® II 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instruction. The sets of PCR primers used were as follows; TLR2: 5'-GGC CAG CAA ATT ACC TGT GTG-3' for forward and 5'-CCA GGT AGG TCT TGG TGT TCA-3' for reverse, TLR4: 5'-TGG ATA CGT TTC CTT ATA AG-3' for forward and 5'-GAA ATG GAG GCA CCC CTT C-3' for reverse, RAGE: 5'-CCT AAT GAG AAG GGA GTA TCT G-3' for forward and 5'-CAC AAG ATG ACC CCA ATG A-3' for reverse; GAPDH: 5'-ACC ACA GTC CAT GCC ATC AC-3' for forward and 5'-TCC ACC ACC CTG TTG CTG TA-3' for

reverse. The thermal cycle contained following profile; denaturation: 94˚C for 1 min, annealing: 56˚C for TLR2, 56˚C for TLR4, 56˚C for RAGE, and 60˚C for GAPDH for 1 min), and extension: 72˚C for 1 min. The PCR products were electrophoresed on agarose gel and stained with ethidium bromide. For semi-quantification, the amounts of mRNAs encoding TLR2, TLR4 and RAGE were estimated by the relative intensity against the intensity of cDNA encoding GAPDH.

Transient transfection of TLR4 siRNA

In preliminary experiments, we optimized the conditions for the efficient transfection of TLR4 siRNA to HGFs. When HGFs were cultured 70 % confluence, the medium was changed to DMEM with 0.5 % FBS and HGFs were transfected with 10μM of either control or TLR4 siRNA in serum-free Opti-MEM Medium (Invitrogen, Carlsbad, CA) by using Lipofectamine® RNA iMAX Reagent (Invitrogen) according to the manufacturer's instructions. After 24 h, the cells were treated with or without calprotectin (50 nM) for indicated times. To determine the efficiency of TLR4 gene silencing, quantitative RT-PCR analysis was performed 24 h after transfection. To examine the cell viability after siRNA transfection, MTT assay was performed as described above.

Intracellular signaling in TLR4 down-regulated HGFs

To examine the intracellular signaling, TLR4 down-regulated HGFs were treated with S100A8, S100A9 and calprotectin (50 nM each) for 30 min. Total cell lysates were extracted with a lysis buffer [10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 % NP-40 and protease inhibitor cocktail (Complete™; Roche Diagnosis, Berkeley, CA)]. The total proteins (10 μg each) were separated in a

denaturing 10 % polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) as described elsewhere. The membranes were then blocked with PVDF Blocking Reagent for CanGet Signal® (Toyobo, Osaka, Japan) and subsequently incubated with appropriate first antibodies. Immuno-reactive proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences) according to the manufacturer's instructions.

Cytokine production

To investigate the effects of calprotectin on the cytokine production, HGFs were treated with S100A8, S100A9 and calprotectin (50 nM each) for 24 hours with or without appropriate inhibitors as described above. Firstly, Pg LPS (1 μg/mL) was used as a control. Furthermore, to examine the effects of TLR4 on calprotectin-mediated cytokine production, TLR4 down-regulated HGFs were also treated with S100A8, S100A9 and calprotectin for 24 hours. Supernatants were collected and stocked at -80 °C until use. The amount of target proteins, IL-1β, IL-6, MCP-1 and TNF-α were measured using sandwich ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

Next, to confirm the effects of TLR4 on calprotectin-mediated cytokine production, HGFs were treated with S100A8, S100A9 and calprotectin (50 nM each) with or without TLR4 inhibitor TAK242 (final concentration: 3 μM) for 12 hours. Total RNA was extracted with RNeasy® Mini Kit (QIAGEN), and first strand cDNA was synthesized by using PrimeScript® II 1st strand cDNA Synthesis Kit (Takara Bio). Quantitative RT-PCR (qRT-PCR) was performed using CFX96™ Real-Time PCR Detection System (Bio-Rad)

with SYBR Green Supermix® (Bio-Rad) on cDNA generated from the reverse transcription of purified RNA. The mRNA expression of target genes was normalized against GAPDH mRNA expression using the comparative cycle threshold method. The sets of PCR primers used were as follows; IL-6: 5'-AGG GCT CTT CGG CAA ATG T-3' for forward and 5'-GAA GAA GGA ATG CCC ATT AAC AAC-3' for reverse, MCP-1: 5'-GCT ATA GAA GAA TCA CCA GCA GCA A-3' for forward and 5'-TGG CCA CAA TGG TCT TGA AG-3' for reverse, GAPDH: 5'-ACC ACA GTC CAT GCC ATC AC-3' for forward and 5'-TCC ACC ACC CTG TTG CTG TA-3' for reverse. Furthermore, supernatants were collected 24 hours later stimulation and stocked at -80 °C until use. In addition, to confirm the effects of TLR4 on calprotectin-mediated cytokine production, HGFs were also treated with S100A8, S100A9 and calprotectin (50 nM each) with or without TLR4 inhibitor VIPER and its control peptide (CP7) (final concentration: 10 μM) for 2 hours. The amount of target proteins, IL-6 and MCP-1 were also measured using sandwich ELISA kits (R&D Systems) according to the manufacturer's instruction.

Statistical analysis

Statistical significances were determined by Student's *t*-test or ANOVA Tukey-HSD analysis. P value less than 0.05 was considered statistically significant. All analyses were performed with SPSS Statistics version 20 (IBM, Chicago, IL).

Results

Effects of calprotectin on cell proliferation

To investigate the effects of calprotectin on cell proliferation, MTT assay was performed (Figure 1). There were no significant differences among S100A8, S100A9 and calprotectin (50 nM, respectively) in the cell proliferation 24-72 h after stimulation (calprotectin: 24 h, P=0.48; 48 h, P=0.95; 72 h, P=0.30, vs None, ANOVA Tukey-HSD). There were also no significant differences among S100A8, S100A9 and calprotectin (100 nM, respectively) in the cell proliferation 24-72 h after stimulation (calprotectin: 24 h, P=0.74; 48 h, P=0.94; 72 h, P=0.90, vs None, ANOVA Tukey-HSD). The cell morphology did not changed throughout the experiments.

TLRs and RAGE mRNA expression in HGFs

To investigate whether HGFs are possible to be a target for calprotectin, expression of TLR2, TLR4 and RAGE mRNA were examined using semi-quantitative RT-PCR (Figure 2). Significant constitutive increase of TLR4 mRNA expression in HGFs was observed compared with oral epithelial cells used as a control ($P<0.001$, Student's *t*-test). Conversely, HGFs showed a little expression of TLR2 and RAGE mRNA compared with oral epithelial cells (TLR2: $P<0.0001$; RAGE: $P=0.006$, Student's *t*-test). Next, expression of both TLR4 and RAGE mRNA did not change by administration of calprotectin into the HGFs culture for 24-48 h.

Induction of several inflammatory cytokines by calprotectin

As shown in Figure 3, calprotectin increased significantly IL-6, MCP-1 and TNF-

α, but not IL-1β production in HGFs (IL-6: P<0.0001; MCP-1: P=0.0002; TNF-α: P=0.0016; IL-1β: P=0.99, vs. None, ANOVA Tukey-HSD). Next, Pg LPS was used as a control. The cytokine productivities of Pg LPS were very weak compared with calprotectin, although Pg LPS increased significantly the cytokine production compared with basal control. Furthermore, S100A9 increased significantly IL-1β and TNF-α production compared to calprotectin (P<0.0001, vs. calprotectin, ANOVA Tukey-HSD). Based on these results, we focused on the calprotectin-mediated IL-6 and MCP-1 production in HGFs.

Effects of NF-κB/ MAPKs inhibitors on calprotectin-mediated IL-6 and MCP-1 production

Both S100A9 and calprotectin increased significantly IL-6 and MCP-1 production in HGFs (P<0.0001, vs. None, ANOVA Tukey-HSD), and there was no significant difference between S100A9 and calprotectin in the cytokines production (IL-6: P=0.99, MCP-1: P=0.21 ANOVA Tukey-HSD) (Figure 4A and 4B). Next, to investigate whether NF-κB or MAPKs play a role in calprotectin-mediated cytokine production, we measured the levels of IL-6 and MCP-1 production in the culture supernatants of HGFs stimulated by calprotectin with or without specific inhibitors. NF-κB inhibition, but not androgen receptor inhibition, significantly suppressed calprotectin-mediated IL-6 and MCP-1 production (IL-6: P<0.0001, calprotectin vs. calprotectin+Bay11-7082; P=0.99, calprotectin vs. calprotectin+Flutamide; MCP-1: P<0.0001, calprotectin vs. calprotectin+Bay11-7082; P=0.99, calprotectin vs. calprotectin+Flutamide ANOVA Tukey-HSD) (Figure 3A and 3B). Next, ERK and p38 MAPK inhibition, but not JNK inhibition, significantly diminished calprotectin-mediated IL-6 production (P=0.047,

calprotectin vs. calprotectin+U0126; P<0.0001, calprotectin vs. calprotectin+SB203580; P=0.99, calprotectin vs. calprotectin+SP600125, ANOVA Tukey-HSD) (Figure 4C). On the other hand, JNK and p38 MAPK inhibition, but not ERK inhibition, significantly diminished calprotectin-mediated MCP-1 production (P=0.066, calprotectin vs. calprotectin+U0126; P<0.0001, calprotectin vs. calprotectin+SB203850; P<0.0001, calprotectin vs. calprotectin+SP600125, ANOVA Tukey-HSD) (Figure 4D). Similar tendency was observed between calprotectin and S100A9 in both IL-6 and MCP-1 production. Induction levels of IL-6 and MCP-1 by S100A8 were a very little compared with S100A9 or calprotectin, although S100A8 increased statistically the cytokines production compared with basal control (IL-6: P=0.0002, MCP-1: P<0.0001, ANOVA Tukey-HSD) (Figure 4A and 4B).

Establishment of *in vitro* systems of TLR4 down-regulation by siRNA in HGFs

To clarify the function of TLR4 in HGFs, we established the TLR4 down-regulating systems by transient siRNA approach. After optimizing conditions for siRNA transfection, we examined the knock down effects of the TLR4-specific siRNA in HGFs transfected with the siRNA using qRT-PCR. As shown in Figure 5A, we confirmed that TLR4 expression was efficiently impaired by transfection with TLR4 siRNA, and the transfection with a control random sequence siRNA did not affect the expression of TLR-4 (P<0.0001, control siRNA vs. TLR4 siRNA, ANOVA Tukey-HSD). Next, the cell cytotoxicity after siRNA transfection was examined using MTT methods. No significant differences were observed between control and TLR4 siRNA-transfected HGFs (P=0.33, control vs. TLR4 siRNA, ANOVA Tukey-HSD) (Figure 5B). These results indicate that we established the TLR4 down-regulated HGFs using siRNA techniques.

Effects of down-regulation of TLR4 by siRNA on calprotectin-induced IL-6 and MCP-1

Both S100A9 and calprotectin increased significantly IL-6 and MCP-1 production in siRNA transfected HGFs (IL-6: P=0.0006, MCP-1: P=0.0043, s100A9 vs. None, IL-6: P=0.0015, MCP-1: P=0.0011, calprotectin vs. None, Student's *t*-test), and there was no significant difference between S100A9 and calprotectin in the cytokines production (IL-6: P=0.31, MCP-1: P=0.84, S100A9 vs. calprotectin, Student's *t*-test) (Figure 5C and 5D). In addition, the significant increases of IL-6 and MCP-1 production by calprotectin were partially, but significantly suppressed in TLR4 down-regulated cells (IL-6: P=0.0002, MCP-1: P=0.0011, cont. vs. TLR4 siRNA, Student's *t*-test). S100A9-mediated IL-6 and MCP-1 production were also partially, but significantly suppressed in TLR4 down-regulated cells (IL-6: P=0.0001, MCP-1: P=0.0080, cont. vs. TLR4 siRNA, Student's *t*-test). S100A8 induced significantly IL-6 and MCP-1 production (IL-6: P=0.019, MCP-1: P=0.0019, vs. None, Student's *t*-test), and the induction of IL-6 production by S100A8 suppressed significantly in TLR4 down-regulated cells (IL-6: P=0.0004, MCP-1: P=0.59, cont. vs. TLR4 siRNA, Student's *t*-test).

Effects of down-regulation of TLR4 by siRNA on calprotectin-mediated intracellular signaling in HGFs

Using TLR4 siRNA transfection system, we examined the calprotectin-mediated intracellular signals in downstream of the TLR4. Phosphorylation of both IκBα and p65 enhanced by calprotectin, and the enhancement diminished by siRNA-mediated down-regulation of TLR4 (Figure 6A). Next, phosphorylation of both ERK and JNK enhanced

dramatically by calprotectin, and the enhancement also diminished by siRNA-mediated down-regulation of TLR4 (Figure 6B). Calprotectin did not induce the phosphorylation of p38 MAPK. The phosphorylation of NF-κB and MAPKs induced by S100A9 resembled the phosphorylation by calprotectin. Furthermore, S100A8 enhanced the phosphorylation of both ERK and JNK, but not p38MAPK, although the S100A8 did not induce the phosphorylation of both IκBα and p65.

Effects of TLR4 inhibitors TAK242 or VIPER on calprotectin-induced IL-6 and MCP-1 production

At first, to confirm the effects of TLR4 on calprotectin-mediated IL-6 and MCP-1 expression, we examined the IL-6 and MCP-1 productivity in HGFs stimulated with calprotectin using TLR4 specific inhibitor TAK242. Calprotectin increased significantly both IL-6 and MCP-1 mRNA expression in HGFs, and the induction of IL-6 and MCP-1 mRNA by calprotectin completely suppressed in HGFs treated with TAK242 (IL-6: P=0.005, MCP-1: P=0.005, with or without TAK242, Student's t-test) (Figure 7A and 7B). Induction of both IL-6 and MCP-1 mRNAs by S100A9 also completely suppressed in HGFs treated with TAK242 (IL-6: P=0.003, MCP-1: P=0.003, with or without TAK242, Student's *t*-test). In addition, slight but statistical induction of both IL-6 and MCP-1 mRNAs by S100A8 also completely inhibited in HGFs treated with TAK242 (IL-6: P=0.0013, MCP-1: P=0.0007, with or without TAK242, Student's *t*-test). Corresponding to the results of qRT-PCR, induction of both IL-6 and MCP-1 proteins by calprotectin completely suppressed in HGFs treated with TAK242 (P<0.0001, with or without TAK242, Student's *t*-test) (Figure 7C and 7D). Induction of both IL-6 and MCP-1 proteins by S100A9 also completely suppressed in HGFs treated with TAK242

(P<0.0001, with or without TAK242, Student's *t*-test). In addition, slight but statistical induction of both IL-6 and MCP-1 proteins by S100A8 completely inhibited in HGFs treated with TAK242 (IL-6: P=0.003, MCP-1: P=0.027, with or without TAK242, Student's *t*-test). Next, we found that induction of both IL-6 and MCP-1 proteins by calprotectin suppressed significantly in HGFs treated with VIPER (P<0.0001, vs. CP7, Student's *t*-test) (Figure 7E and 7F). Induction of both IL-6 and MCP-1 proteins by S100A9 also suppressed significantly in HGFs treated with VIPER (P<0.0001, vs. CP7, Student's *t*-test). In addition, induction of both IL-6 and MCP-1 proteins by S100A8 also inhibited significantly in HGFs treated with VIPER (IL-6: P=0.0023, MCP-1: P=0.013, vs. CP7, Student's *t*-test). Slight but statistical induction of both IL-6 and MCP-1 proteins by Pg LPS inhibited significantly in HGFs treated with VIPER (IL-6: P=0.046, MCP-1: P=0.014, vs. CP7, Student's *t*-test).

Discussion

Calprotectin, heterodimers of S100A8 and S100A9, is an abundant cytosolic protein of leukocytes (especially neutrophils) that is released during inflammation (Kostakis, 2013). In general, it is well-known that calprotectin plays an important role in the host defense against microorganisms by infiltration of neutrophils (Burri, 2014) or its direct antimicrobial effects (Loomans, 1998). Recently, we have reported that elevated calprotectin levels in GCFs of periodontitis patients are useful clinically as a reliable inflammatory marker for diagnosis of the periodontitis (Kido, 1999), although the biological functions of exogenous calprotectin in inflamed periodontal lesions are unclear.

Previous report has demonstrated that S100A9 promoted cell growth and the secretion of IL-6, IL-8 and collagen through RAGE signaling in human lung fibroblasts (Xu, 2013). Furthermore, it has been shown that S100A8 interacts with RAGE and increases the proliferation of anaplastic thyroid carcinoma cells by activating p38, ERK1/2 and JNK signaling pathways (Reeb, 2015). Therefore, S100A8 and S100A9 have dependent or independent functions, and these functions could be regulated at least in part by different mechanisms (Wang L, 2014). In our results, since we demonstrated the proliferative activities of HGFs were not changed 24 hours after stimulation of S100A8, S100A9 and calprotectin (50, 100 nM, respectively) (Figure 1), we selected the 50 nM of calprotectin as a suitable concentration throughout experiments. Furthermore, interestingly, no significant differences were observed in the cell proliferative activity of HGFs treated with calprotectin for 48-72 hours. Although the discrepancy of the cell proliferation between lung fibroblasts and HGFs might be explained by the difference of cell types, further experiments will be needed to clarify the mechanisms.

Both TLR4 and RAGE are thought to be important receptors for calprotectin (Catalán, 2011; Chernov, 2015; Wang L, 2014). It has been reported that calprotectin induces cell migration and invasion through p38 MAPK dependent NF-κB activation in gastric cancer (Kwon, 2013). In addition, Narumi et al (2015) has been reported that natural killer (NK) cells are activated in *via* the calprotectin-RAGE signaling, and the calprotectin-induced NK cell activation may be useful for cancer immunotherapy. Importantly, we found that human oral epithelial cell line TR146 constitutively express both TLR4 mRNA and RAGE mRNA, whereas, HGFs express TLR4 mRNA constitutively, but not RAGE mRNA (Figure 2). These results indicate that TLR4 is possible to be a main target receptor for calprotectin in HGFs. Calprotectin-mediated TLR4 signaling in HGFs might play an important role in the activation of transcription factor NF-κB, which controls the expression of several inflammatory cytokine genes such as IL-6 and MCP-1.

Pro-inflammatory cytokines such as IL-1, IL-6, MCP-1 and TNF are believed to be the major pathological mediators of inflammatory diseases (Okada, 1998). Previously, Ehlermann et al (2006) reported that calprotectin had much more effective than homodimers of S100A8 or S100A9 in the expression of IL-6 and MCP-1 production in advanced glycation end products (AGE)-mediated human umbilical venous endothelial cells (HUVECs). Whereas, we have demonstrated that both S100A9 and calprotectin increased significantly IL-6, MCP-1 and TNF-α production in HGFs, and there was no significant difference between S100A9 and calprotectin in the cytokines production. Furthermore, we have found that induction of IL-6, MCP-1 and TNF-α by S100A8 was a very little compared with S100A9 or calprotectin, although S100A8 increased the cytokines production statistically compared with the basal control. A little elevation of IL-6, MCP-1 and TNF-α production by S100A8 is not significant patho-physiologically,

and might not lead cell-cell interactions through several cytokine cascades of the downstream. Further experiments will be needed to clarify the clinical significance of S100A8 in the future. Interestingly, we found that S100A9 increased significantly IL-1β and TNF-α production compared with calprotectin in HGFs. These results indicate that roles of calprotectin should be different from S100A9 in pathophysiology of periodontal diseases. We have considered that calprotectin effects might be dependent on the binding affinity of S100A9 to the membrane bound receptors in HGFs. In addition, LPS is a well-known factor to activate TLR2/4 signaling in various cells (Rossol, 2011). We investigated whether LPS can be substituted for calprotectin in IL-6 and MCP-1 production from HGFs. In the present study, LPS derived from Pg (Pg LPS) was used because our objective is to clarify the pathophysiology of periodontal diseases. Interestingly, we found that the levels of cytokine production by Pg LPS (1 μg/mL) were very weak compared with calprotectin (50 nM). Pg LPS might not be substituted for calprotectin in IL-6, MCP-1 and TNF-α production from HGFs. These results encourage that calprotectin should be an attractive target for periodontal diseases.

IL-6 is an important pro-inflammatory cytokine that elicits multifactorial activities in various cell types (Kishimoto, 1989), and sIL-6R has agonistic functions of IL-6 and requires cells expressing the gp130 (Jones, 2002). We have reported previously that IL-6 induces sIL-6R release significantly in PMA-differentiated THP-1 macrophages (Sawada et al, 2013). Macrophages exiting in periodontitis lesions would be a candidate of sIL-6R-producing cells. Although HGFs do not express surface IL-6R to respond to the IL-6 (Naruishi, 1999), calprotectin-induced IL-6 in HGFs would respond to themselves by autocrine mechanism in the presence of sIL-6R released from macrophages (Figure 8). Since IL-6 increases MMP-1, cathepsin L and VEGF production in HGFs (Sawada, 2013),

calprotectin-induced IL-6 production in HGFs may lead in periodontitis progression through fibroblasts-macrophages cross-talk. MCP-1 is an also important chemokine that induces the infiltration of immune cells such as neutrophils, lymphocytes and macrophages (Faller, 1997), leading to chronic inflammation. Therefore, cellular responses to elevated IL-6 and MCP-1 mediated by calprotectin would contribute to the development of periodontitis.

Next, to investigate the intracellular regulation for IL-6 and MCP-1 production in HGFs treated by calprotectin, we performed several *in vitro* experiments using inhibitors. Importantly, we found that Bay11-7082, an IKK complex inhibitor/ potent suppressor of NF-κB activation, suppressed significantly IL-6 and MCP-1 production in HGFs. NF-κB is known to be regulated by several intracellular molecules including MAPKs family, ERK, JNK and p38 MAPK (Lee, 2011). Together with NF-κB inhibitor, we found the MAPK inhibitors have been shown to partially inhibit calprotectin-mediated the cytokine production. In general, there are no relationship between TLR signals and androgen receptor signals. We used a selective antagonist of the androgen receptor competing with androgens such as testosterone, flutamide, as a negative control of TLR signals. Since flutamide did not inhibit the cytokine production, the significance of calprotectin-mediated NF-κB and MAPK signaling, directly or indirectly, might have been emphasized in the IL-6 and MCP-1 production in HGFs. Most importantly, we demonstrated that calprotectin-mediated IL-6 and MCP-1 production was significantly suppressed in TLR4 down-regulated HGFs established using siRNA approach. This regulation indicates that activation of TLR4 signaling by calprotectin is an important pathway for the cytokine production in HGFs, because HGFs express TLR4, but not RAGE. In addition, it is still unknown whether TLR4 is involved in activation of

calprotectin-induced NF-κB and MAPKs in HGFs. Importantly, we found that calprotectin enhanced a phosphorylation of both NF-κB (IκBα and p65) and MAPKs (ERK, JNK and p38 MAPK), and the calprotectin-mediated phosphorylation of NF-κB and MAPKs was dramatically inhibited in TLR4 down-regulated HGFs. These results have shown that the activation of both NF-κB and MAPKs signaling by calprotectin might be strongly affected by TLR4 down-regulation in HGFs. Finally, using TLR4 specific inhibitor TAK242, we have confirmed whether calprotectin-mediated IL-6 and MCP-1 production was suppressed in HGFs by inhibition of TLR4. As expected, the induction of IL-6 and MCP-1 by calprotectin was completely suppressed by TAK242 in both mRNAs and proteins expression levels. Furthermore, we demonstrated that the induction of IL-6 and MCP-1 by calprotectin was significantly suppressed by another TLR4 specific inhibitor VIPER. These results support that HGFs express TLR4 as a target receptor for calprotectin, and a series of results indicate that TLR4 is an important target for IL-6 and MCP-1 production in HGFs treated with calprotectin.

In recent years, immune-based therapies for cancer by targeting TLRs are generating substantial interest because of the success of immune regulators (Patra, 2016). TLRs inhibition might be useful for treatment effects in various inflammatory diseases such as rheumatoid arthritis and periodontitis. There is a lot of reports focused on understanding how cytokines are released from HGFs and how they cause beneficial or harmful effects in inflamed periodontal lesions. Fibroblast-mediated inflammation may be also regulated by calprotectin through TLR4 signaling, suggesting one of the potential mechanisms in the progression of periodontitis. Taken together, calprotectin/TLR4 signaling in HGFs promotes inflammatory cascades through IL-6 and MCP-1 production. This pathway could be an attractive therapeutic target for periodontal diseases.

Acknowledgements

This study was supported by Grant-in-Aid for Scientific Research (B) (No. 15H05054), Scientific Research (C) (No. 16K11832) and Grant-in-Aid for Young Scientists (B) (No. 15K20621) from the Japan Society for the Promotion of Science.

Conflict of Interest

The authors have no conflict of interest.

References

Bao Y, Cao X. 2014. The immune potential and immunopathology of cytokine-producing B cell subsets: a comprehensive review. J Autoimmun 55:10-23.

Beklen A, Ainola M, Hukkanen M, Gürgan C, Sorsa T, Konttinen YT. 2007. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. J Dent Res 86:347-351.

Blair HC, Athanasou NA. 2004. Recent advances in osteoclast biology and pathological bone resorption. Histol Histopathol 19:189-199.

Burri E, Beglinger C. 2014. The use of fecal calprotectin as a biomarker in gastrointestinal disease. Expert Rev Gastroenterol Hepatol. 8:197-210.

Catalán V, Gómez-Ambrosi J, Rodríguez A, Ramírez B, Rotellar F, Valentí V, Silva C, Gil MJ, Fernández-Real JM, Salvador J, Frühbeck G. 2011. Increased levels of calprotectin in obesity are related to macrophage content: impact on inflammation and effect of weight loss. Mol Med 17:1157-1167.

Cesaro A, Anceriz N, Plante A, Pagé N, Tardif MR, Tessier PA. 2012. An inflammation loop orchestrated by S100A9 and calprotectin is critical for development of arthritis. PLoS One 7:e45478.

Chernov AV, Dolkas J, Hoang K, Angert M, Srikrishna G, Vogl T, Baranovskaya S, Strongin AY, Shubayev VI. 2015. The calcium-binding proteins S100A8 and S100A9 initiate the early inflammatory program in injured peripheral nerves. J Biol Chem 290:11771-11784.

Ehlermann P, Eggers K, Bierhaus A, Most P, Weichenhan D, Greten J, Nawroth PP, Katus HA, Remppis A. 2006. Increased proinflammatory endothelial response to

S100A8/A9 after preactivation through advanced glycation end products. Cardiovasc Diabetol 5:6.

Faller DV, Weng H, Graves DT, Choi SY. 1997. Moloney murine leukemia virus long terminal repeat activates monocyte chemotactic protein-1 protein expression and chemotactic activity. J Cell Physiol 172:240-252.

Graves DT, Cochran D. 2003. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol 74:391-401.

Gupta M, Chaturvedi R, Jain A. 2013. Role of monocyte chemoattractant protein-1 (MCP-1) as an immune-diagnostic biomarker in the pathogenesis of chronic periodontal disease. Cytokine 61:892-897.

Jones SA, Rose-John S. 2002. The role of soluble receptors in cytokine biology: the agonistic properties of the sIL-6R/IL-6 complex. Biochim Biophys Acta 1592:251-263.

Kido J, Nakamura T, Kido R, Ohishi K, Yamauchi N, Kataoka M, Nagata T. 1999. Calprotectin in gingival crevicular fluid correlates with clinical and biochemical markers of periodontal disease. J Clin Periodontol 26:653-657.

Kishimoto T.1989. The biology of interleukin-6. Blood 74:1-10.

Kostakis ID, Cholidou KG, Vaiopoulos AG, Vlachos IS, Perrea D, Vaos G. 2013. Fecal calprotectin in pediatric inflammatory bowel disease: a systematic review. Dig Dis Sci 58:309-319.

Kwon CH, Moon HJ, Park HJ, Choi JH, Park do Y. 2013. S100A8 and S100A9 promotes invasion and migration through p38 mitogen-activated protein kinase-dependent NF-κB activation in gastric cancer cells. Mol Cells 35:226-234.

Lee CW, Lin CC, Lee IT, Lee HC, Yang CM. 2011. Activation and induction of cytosolic phospholipase A2 by TNF-α mediated through Nox2, MAPKs, NF-κB, and p300 in human tracheal smooth muscle cells. J Cell Physiol 226:2103-2114.

Loomans HJ, Hahn BL, Li QQ, Phadnis SH, Sohnle PG. 1998. Histidine-based zinc-binding sequences and the antimicrobial activity of calprotectin. J Infect Dis 177:812-814.

Naruishi K, Nishimura F, Yamada-Naruishi H, Omori K, Yamaguchi M, Takashiba S. 2003. C-*jun* N-terminal kinase (JNK) inhibitor, SP600125, blocks interleukin (IL)-6-induced vascular endothelial growth factor (VEGF) production: cyclosporine A partially mimics this inhibitory effect. Transplantation 76:1380-1382.

Naruishi K, Takashiba S, Chou HH, Arai H, Nishimura F, Murayama Y. 1999. Role of soluble interleukin-6 receptor in inflamed gingiva for binding of interleukin-6 to gingival fibroblasts. J Periodontal Res 34:296-300.

Narumi K, Miyakawa R, Ueda R, Hashimoto H, Yamamoto Y, Yoshida T, Aoki K. 2015. Proinflammatory Proteins S100A8/S100A9 Activate NK Cells via Interaction with RAGE. J Immunol 194:5539-5548.

Nisapakultorn K, Ross KF, Herzberg MC. 2001. Calprotectin expression in vitro by oral epithelial cells confers resistance to infection by Porphyromonas gingivalis. Infect Immun 69:4242-4247.

Okada H, Murakami S. 1998. Cytokine expression in periodontal health and disease. Crit Rev Oral Biol Med 9:248-266.

Patra MC, Choi S. 2016. Recent progress in the development of Toll-like receptor (TLR) antagonists. Expert Opin Ther Pat 26:719-730.

Reeb AN, Li W, Sewell W, Marlow LA, Tun HW, Smallridge RC, Copland JA, Spradling K, Chernock R, Lin RY. 2015. S100A8 is a novel therapeutic target for anaplastic thyroid carcinoma. J Clin Endocrinol Metab 100:E232-242.

Rossol M, Heine H, Meusch U, Quandt D, Klein C, Sweet MJ, Hauschildt S. 2011. LPS-induced cytokine production in human monocytes and macrophages. Crit Rev Immunol 31: 379-446.

Sawada S, Chosa N, Ishisaki A, Naruishi K. 2013. Enhancement of gingival inflammation induced by synergism of IL-1β and IL-6. Biomed Res 34:31-40.

Steinbakk M, Naess-Andresen CF, Lingaas E, Dale I, Brandtzaeg P, Fagerhol MK. 1990. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. Lancet 336:763-765.

Takashiba S, Naruishi K, Murayama Y. 2003. Perspective of cytokine regulation for periodontal treatment: fibroblast biology. J Periodontol 74:103-110.

Wang L, Luo H, Chen X, Jiang Y, Huang Q. 2014. Functional characterization of S100A8 and S100A9 in altering monolayer permeability of human umbilical endothelial cells. PLoS One 9:e90472.

Xu X, Chen H, Zhu X, Ma Y, Liu Q, Xue Y, Chu H, Wu W, Wang J, Zou H. 2013. S100A9 promotes human lung fibroblast cells activation through receptor for advanced glycation end-product-mediated extracellular-regulated kinase 1/2, mitogen-activated protein-kinase and nuclear factor-κB-dependent pathways. Clin Exp Immunol 173:523-535.

Yamaguchi T, Naruishi K, Arai H, Nishimura F, Takashiba S. 2008. IL-6/sIL-6R enhances cathepsin B and L production via caveolin-1-mediated JNK-AP-1 pathway in human gingival fibroblasts. J Cell Physiol 217:423-432.

Zheng Y, Hou J, Peng L, Zhang X, Jia L, Wang X, Wei S, Meng H. 2014. The pro-apoptotic and pro-inflammatory effects of calprotectin on human periodontal ligament cells. PLoS One 9:e110421.

Figure Legends

Figure 1. Effects of calprotectin on cell proliferation

After HGFs were treated with S100A8, S100A9 and calprotectin (50, 100 nM, respectively) for 24-72 h, the cell proliferative activity was determined by MTT assay. Data represents as the mean ± SD from 3 independent experiments. CPT, calprotectin. NS, not significant differences (ANOVA Tukey-HSD).

Figure 2. TLRs and RAGE mRNA expression in HGFs

(A) HGFs were cultured and total RNA was prepared as described in "Materials and Methods", and analyzed to semi-quantitative RT-PCR analysis using specific primer for TLR2, TLR4 and RAGE. Human oral epithelial cells (HOEC) was used as a control cell. Relative density of amplified cDNA encoding TLR4 (B) and RAGE (C) was calculated and expressed as a ratio against that encoding GAPDH. (D) HGFs were treated with 50 nM calprotectin for 24-48 h, and analyzed to semi-quantitative RT-PCR analysis using specific primer for TLR4 and RAGE. Relative density of amplified cDNA encoding TLR4 (E) and RAGE (F) was calculated and expressed as a ratio against that encoding GAPDH. Data are expressed as means ± SD of three independent experiments. CPT, calprotectin. **, $P<0.01$ (Student's *t* test); NS, not significant differences (ANOVA Tukey-HSD).

Figure 3. Production of inflammatory cytokines in HGFs treated with calprotectin

The levels of target molecules (IL-1β, TNF-α, IL-6 and MCP-1) were measured using ELISA Kits. HGFs were treated with S100A8, S100A9 and calprotectin (50 nM each) for

24 h. Pg LPS (1 μg/mL) was used as a control. Data represents as the mean ± SD from 3 independent experiments. CPT, calprotectin; Pg, *Porphyromonas gingivalis*. **, P<0.01, as compared with control (ANOVA Tukey-HSD).

Figure 4. IL-6 and MCP-1 production in HGFs treated with calprotectin and the effects of several inhibitors on the productivity

The levels of target molecules (IL-6 and MCP-1) were measured using ELISA Kits. HGFs were pretreated with IKK inhibitor Bay11-7082 (50 μM) for 24 h or androgen receptor inhibitor flutamide (10 μM) for 1 h and treated with S100A8, S100A9 and calprotectin (50 nM each) for 24 h. (A) IL-6 levels. (B) MCP-1 levels. HGFs were pretreated with ERK inhibitor U0126 (10 μM), JNK inhibitor SP600125 (10 μM) and p38MAPK inhibitor SB2013580 (10 μM) for 1 h and treated with S100A8, S100A9 and calprotectin (50 nM each) for 24 h. (C) IL-6 levels. (D) MCP-1 levels. Data represents as the mean ± SD from 3 independent experiments. CPT, calprotectin. **, P<0.01, *, P<0.05 as compared with control (ANOVA Tukey-HSD).

Figure 5. Transient transfection of TLR4 siRNA in HGFs and inhibitory effects of down-regulation of TLR4 on calprotectin-induced IL-6 and MCP-1 production

HGFs were transfected with the siRNA targeted against TLR4 or a random sequence (control siRNA). (A) Expression of TLR4 mRNA. Cells were transfected with siRNA, then harvested 24 h after transfection. The mRNA levels were analyzed using qRT-PCR methods as described in “Materials and Methods” section. Data represents as the mean ± SD from 2 independent experiments. **, P<0.01 as compared with control (ANOVA Tukey-HSD). (B) Cell cytotoxicity by siRNA transfection. HGFs were transfected with

TLR4 or control siRNA and cells were cultured for 24 h. The cell cytotoxicity was determined by MTT assay as described in "Materials and Methods" section. Data represents as the mean ± SD from 2 independent experiments. NS, not significant differences (ANOVA Tukey-HSD). HGFs were transfected with the siRNA targeted against TLR4 or a random sequence for 24 h, and the cells were treated with S100A8, S100A9 and calprotectin (50 nM each) for 24 h. The levels of target molecules (IL-6 and MCP-1) were measured using ELISA Kits. (C) IL-6 levels. (D) MCP-1 levels. Data represents as the mean ± SD from 3 independent experiments. **, $P<0.01$ as compared with control (Student's *t*-test).

Figure 6. Inhibitory effects of down-regulation of TLR4 on calprotectin-induced intracellular signals

HGFs were transfected with the siRNA targeted against TLR4 or a random sequence for 24 h, and the cells were treated with S100A8, S100A9 and calprotectin (50 nM each) for 30 min. Signaling molecules were detected by Western blotting. Cell lysates were resolved by SDS-PAGE and analyzed using Western blotting probed with antibodies against (A) phospho-p65, phospho-IκBα, (B) phospho-ERK, phospho-JNK and phospho-p38MAPK. Equal loading of total lysates (10 μg) was confirmed by re-probing with antibodies against each molecule (A) p65, IκBα, (B) ERK, JNK and p38MAPK. This figure shows a result of representative 3 independent experiments. Quantitation of the phosphorylated protein levels was performed by densitometric scanning of each band using Image J software (NIH, Washington DC, USA), and fold changes of each band were expressed as a ratio of control.

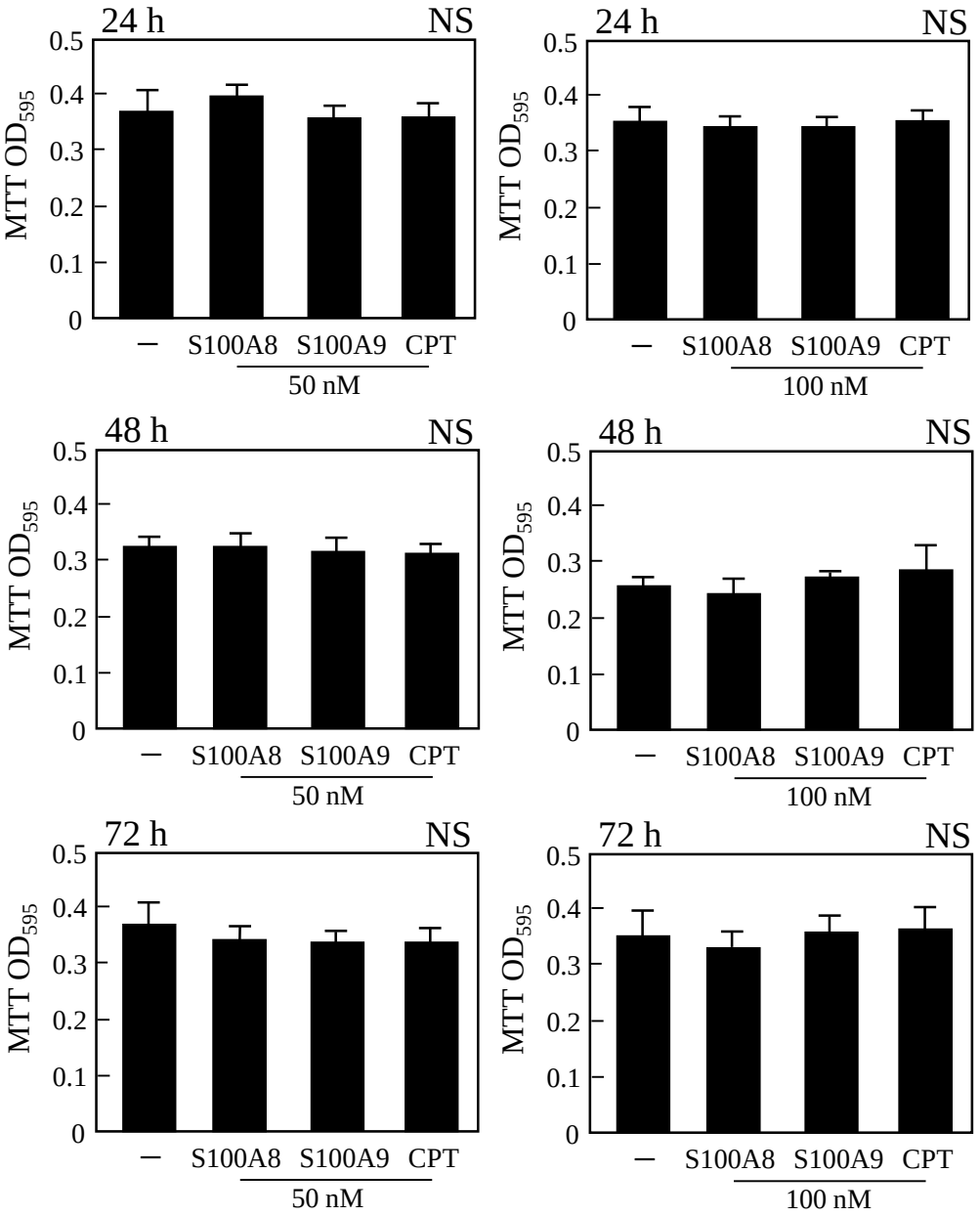
Figure 7. Effects of TLR4 inhibitors TAK242 or VIPER on calprotectin-induced IL-6 and MCP-1 production

HGFs were pretreated with TLR4 inhibitor TAK242 (3 μM) for 12 h and treated with S100A8, S100A9 and calprotectin (50 nM each). Total RNAs were collected after 12 hours later, and the mRNA levels were analyzed using qRT-PCR methods as described in "Materials and Methods" section. Supernatants were collected after 24 hours later, and the protein levels of target molecules (IL-6 and MCP-1) were measured using ELISA Kits. Expression of IL-6 (A) and MCP-1 (B) mRNA. Production of IL-6 (C) and MCP-1 (D) proteins. Next, HGFs were also pretreated with TLR4 inhibitor VIPER (final: 10 μM) or its control peptide CP7 (10 μM) for 2 h and treated with S100A8, S100A9, calprotectin (50 nM each) and 1 μg/mL Pg LPS. Supernatants were collected after 24 hours later, and the protein levels of target molecules (IL-6 and MCP-1) were measured using ELISA Kits. Production of IL-6 (E) and MCP-1 (F) proteins. Data represents as the mean ± SD from 2 independent experiments. LPS, Pg LPS. **, $P<0.01$, *, $P<0.05$ as compared with control (Student's *t*-test).

Figure 8. Schematic representation of HGFs/ macrophages cross-talk in periodontitis lesions

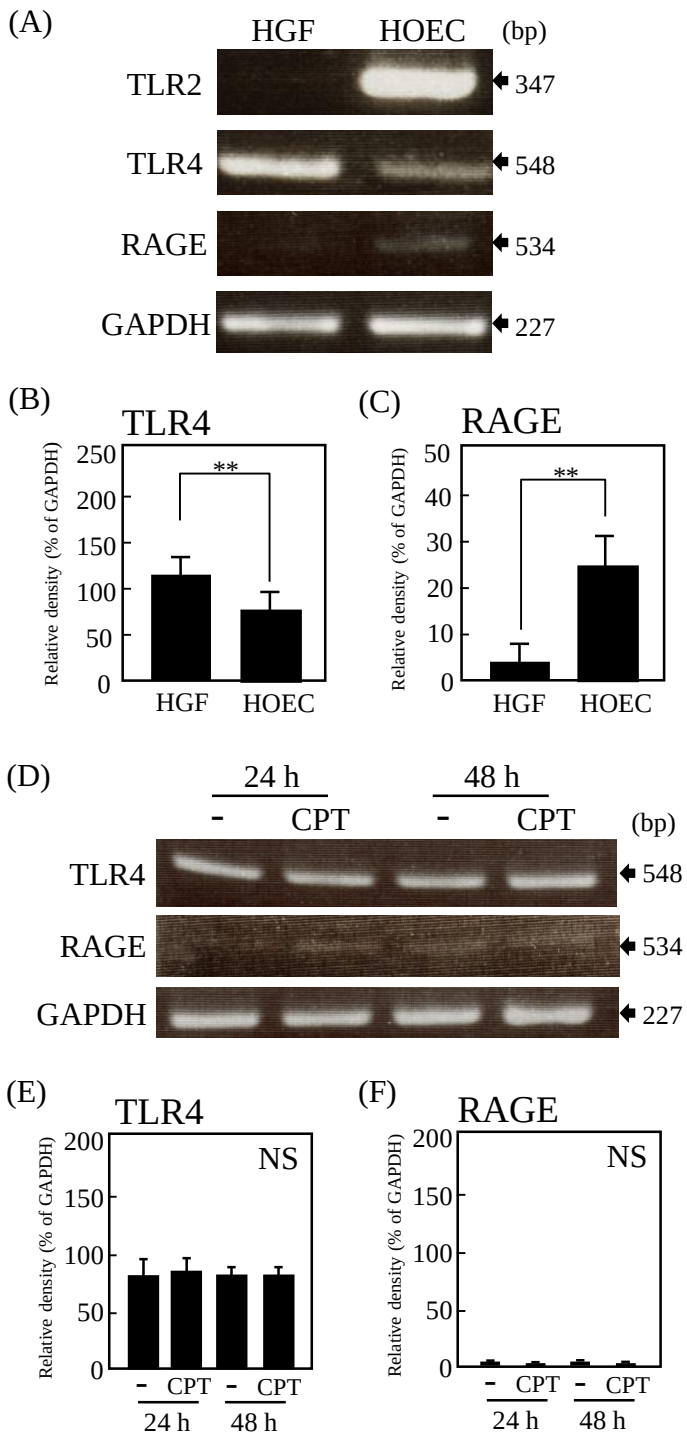
Calprotectin up-regulates IL-6 responsiveness of HGFs by autocrine/ paracrine loops, resulting in progression of periodontitis.

Figure 1



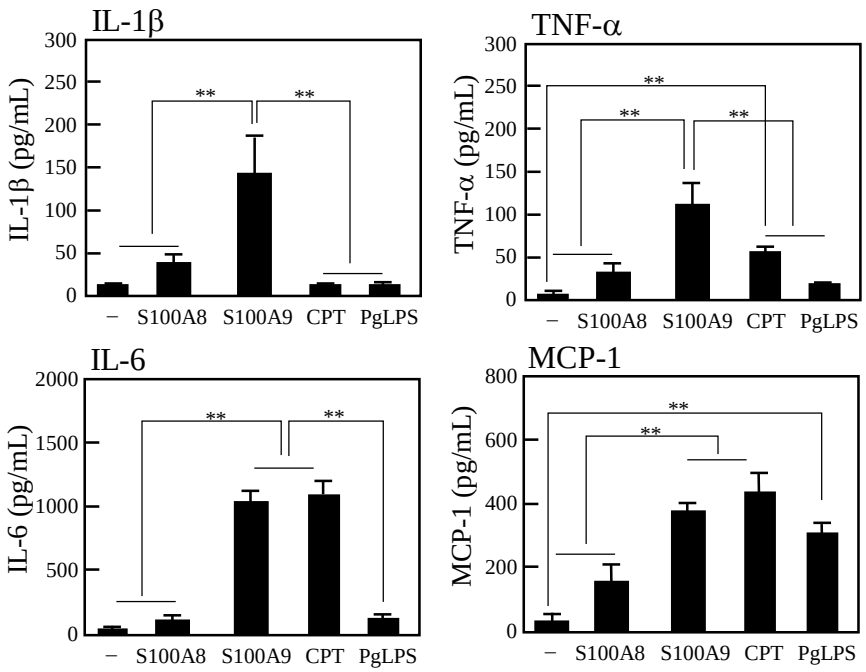
Nishikawa et al.

Figure 2



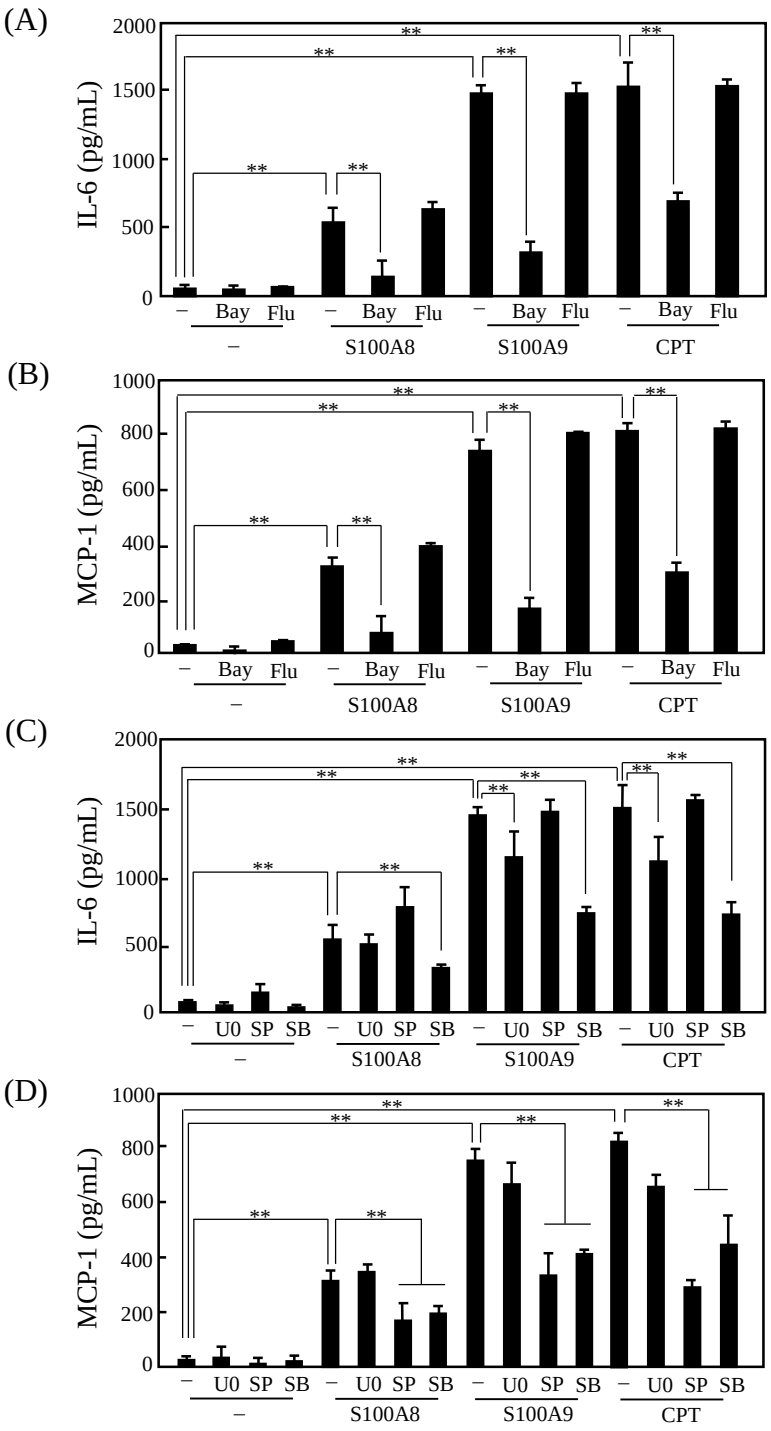
Nishikawa et al.

Figure 3



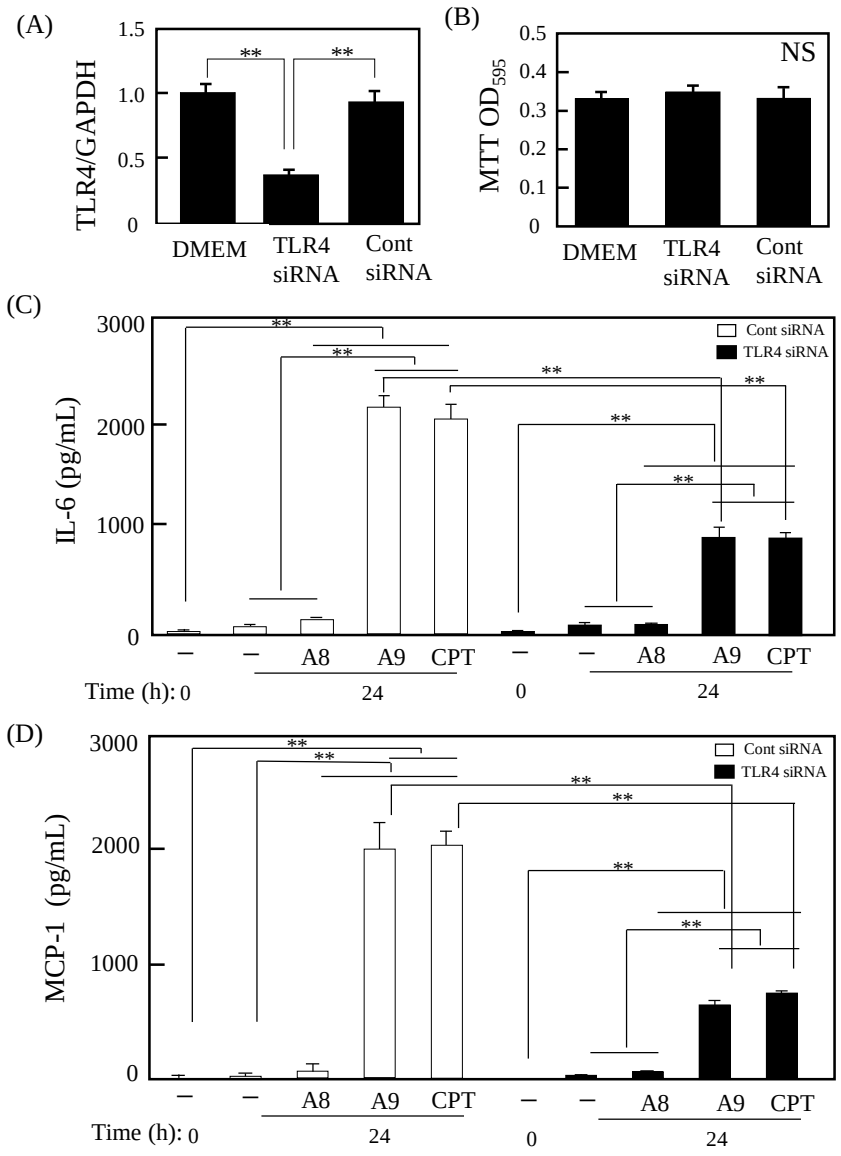
Nishikawa et al.

Figure 4



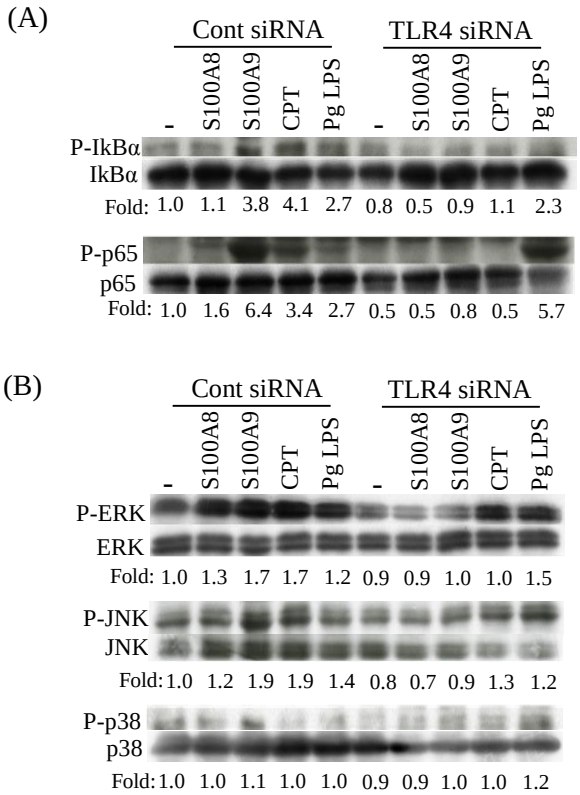
Nishikawa et al.

Figure 5



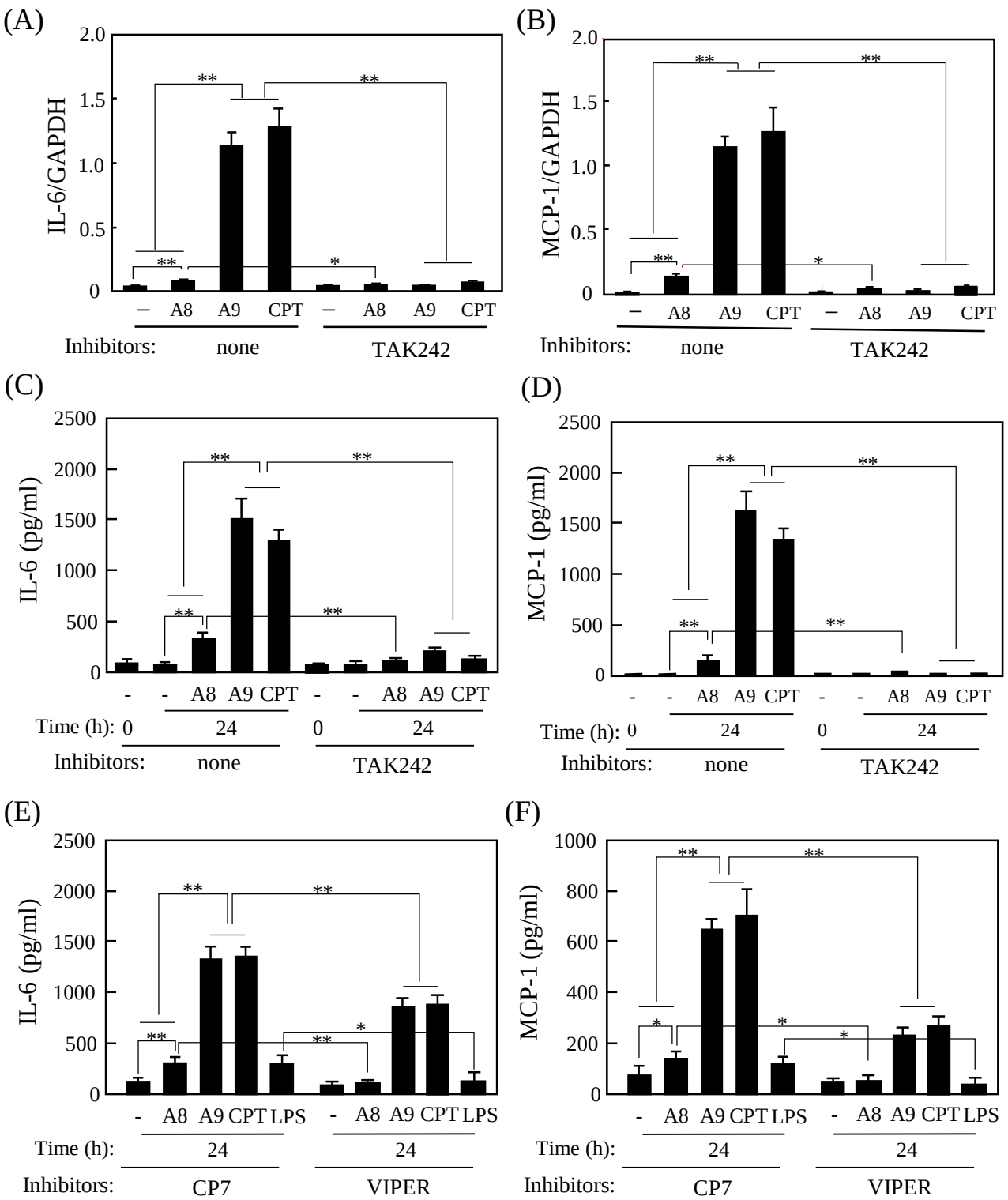
Nishikawa et al.

Figure 6



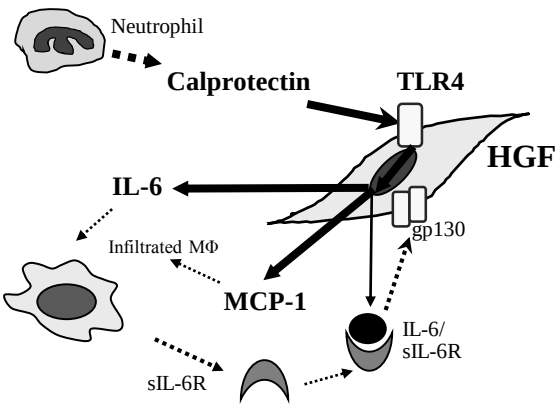
Nishikawa et al.

Figure 7



Nishikawa et al.

Figure 8



Nishikawa et al.