TITLE:

Cyclosporin A and Phenytoin Modulate Inflammatory Responses.

Short title: Cyclosporin A, Phenytoin and TLR Signaling

Key words: cyclosporin A, phenytoin, gingival overgrowth, inflammation, TLR

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ABSTRACT

Gingival overgrowth is a common side effect of administration of the immunosuppressant cyclosporin A and the anti-epileptic drug phenytoin. While cyclosporin-induced gingival overgrowth is often accompanied by gingival inflammation, phenytoin-induced gingival overgrowth usually forms fibrotic lesions. To determine whether these drugs alter the inflammatory responses of gingival fibroblasts, we investigated the effects of cyclosporin and phenytoin on Toll-like receptor (TLR)-mediated responses to microbial components. In Chinese hamster ovary reporter cell lines, cyclosporin alone triggered signaling, whereas phenytoin down-regulated signaling induced by TLR2 or TLR4 ligand. In human gingival fibroblasts, cyclosporin alone did not induce evident inflammatory responses but augmented the expression of CD54 and the production of interleukin (IL)-6 and IL-8 induced by TLR ligands, whereas phenytoin attenuated those responses. Cyclosporin also augmented CD54 expression in gingiva of mice injected with lipopolysaccharide. These results indicated that cyclosporin positively and phenytoin negatively modulated inflammatory responses of human gingival fibroblasts.

INTRODUCTION

Cyclosporin A is a drug prescribed to prevent rejection of solid organ transplants and for the treatment of some autoimmune diseases (Wondimu *et al*., 1997). The immunosuppressive effect of cyclosporin is attributed to down-regulation of the transcription factor NFAT through inhibition of calcineurin, repressing the production of interleukin (IL)-2 in T cells (Chow *et al*., 1999). Phenytoin is an anti-epileptic drug used to treat seizures by blocking the voltage-gated sodium channels in neural cells (Segal and Douglas, 1997). Both drugs are known to be often associated with enlargement of gingival tissue (Kataoka *et al*., 2005).

The characteristics of gingival overgrowth caused by these drugs are different. While cyclosporin-induced gingival overgrowth is often described as a relatively inflamed lesion with increased number of microvessels and inflammatory cells (Kantarci *et al*., 2007; Bullon *et al*., 2007), phenytoin-induced gingival overgrowth is characterized by fibrotic tissue (Uzel *et at*., 2001; Trackman *et al*., 2004; Kantarci *et al*., 2007).

The presence of dental plaque is often associated with gingival overgrowth (Seymour *et al*., 2000). Morphological changes in gingiva lead to the retention of dental plaque. The bacterial components in dental plaque can be recognized by Toll-like receptors (TLRs), which are sensors of pathogen-associated molecular patterns (Kawai and Akira, 2006). Human gingival fibroblasts (HGFs) constitutively express TLRs and can respond to microbial components. Recognition of the cell wall components peptidoglycan and lipoproteins by TLR2 and recognition of the outer membrane component lipopolysaccharide (LPS) by TLR4 lead to a series of events, including nuclear factor

(NF)-κB activation, that result in cytokine production and expression of adhesion molecules in HGFs, which play an important role in inflammatory response in periodontal tissue (Mahanonda *et al.*, 2007).

The aim of this study was to determine whether cyclosporin and phenytoin can modulate the inflammatory responses of HGFs to microbial components, such as lipoproteins and LPS.

MATERIALS & METHODS

Reagents

Cyclosporin A and phenytoin were obtained from Wako (Osaka, Japan). For *in vivo* experiments, cyclosporin was purchased from Novartis (Basel, Switzerland). Anti-CD25 monoclonal antibody conjugated with fluorescein isothiocyanate (FITC) was obtained from Becton Dickinson (Bedford, MA). Anti-CD54 monoclonal antibody conjugated with FITC was obtained from Beckman Coulter (Fullerton, CA) and anti-CD54 polyclonal antibody was obtained from Protein Tech Group (Chicago, IL). Anti-Flag polyclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO). Non-specific rabbit immunoglobulin was purchased from DakoCytomation (Glostrup, Denmark). A synthetic lipid A, compound 506, was purchased from Peptide Institute (Osaka, Japan). Ultra pure *Escherichia coli* LPS and a synthetic lipopeptide, Pam_3CSK_4, were obtained from Invivogen (San Diego, CA). Enzyme-linked immunosorbent assay (ELISA) kits, DuoSet, for IL-6 and IL-8 and MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay kit were obtained from R&D Systems (Minneapolis, MN).

Cell culture

The engineering of the Chinese hamster ovary (CHO) reporter cell line CHO/CD14 has been previously described in detail (Delude *et al.*, 1998). This clonal line expresses surface CD25 antigen under the control of a region from the human E-selectin promoter containing the NF-κB binding site. CHO/CD14 cells were transfected with the cDNA for human TLR2 with a Flag tag or the cDNA for human TLR4 with a Flag tag, and the

resulting clones were named CHO/CD14/TLR2 and CHO/CD14/TLR4, respectively (Lien *et al*., 1999).

HGFs were isolated from seven healthy donors (4 males, 3 females; mean age: 52 ± 12.4 years) following the method described previously (Hayashi *et al*., 1994). Informed consent was obtained from each patient prior to sampling, and the experimental protocol was approved by the Ethics Committee in Nagasaki University.

The cells were grown as adherent monolayers in conditioned media supplemented with 10% fetal bovine serum unless otherwise mentioned.

Treatment of mice and immunohistochemistry

Twenty-four male BALB/c mice were purchased from Clea Japan (Tokyo, Japan). Animal care and experimental procedures were in accordance with the Guidelines for Animal Experimentation of Nagasaki University. Mice were divided into three groups and each group received intraperitoneal injections of either 40 mg/kg of cyclosporin, 40 mg/kg of phenytoin or phosphate-buffered saline (PBS) every 24 hrs for 5 days. Two hours after each intraperitoneal injection, five mice of each group received injections of 1 µg of *E. coli* LPS in 3 µl of PBS under ether anesthesia; each injection was delivered into the mesial gingiva of the first molar of the left mandible. Three mice of each group received PBS injection. Mice were sacrificed 24 hrs after the fifth injection and left mandibles were removed. Tissues were prepared as previously reported (Ozaki *et al*., 2009) and they were subjected to immunohistochemical staining of CD54. Non-specific rabbit immunoglobulin was used for control staining. The number of CD54-positive fibroblast-like cells and the number of total fibroblast-like cells were counted in four

areas of 250 x 250 μm, 250 μm from the bone surface so that the areas would have high concentrations of fibroblasts exposed to LPS. Positively stained vascular endothelium was used as control.

Cytokine assays

HGFs from seven individuals were plated at a density of 5×10^4 cells *per* well in a 96-well plate. Confluent monolayers of HGFs were stimulated with lipid A or Pam_3CSK_4 in the presence or absence of cyclosporin or phenytoin.   Following incubation for 18 hrs, cell-free supernatants were harvested and analyzed for IL-6 and IL-8 release by ELISA.

Flow cytometric analysis

CHO transfectants were plated in a 24-well plate at a density of 10^5 cells *per* well and HGFs from seven individuals were plated in a 6-well plate at a density of 2×10^5 cells *per* well. After overnight incubation, confluent monolayers of cells remained unstimulated or were stimulated with ultra pure LPS, lipid A or Pam_3CSK_4 in the presence or absence of cyclosporin or phenytoin. Following incubation for 18 hrs, the cells were stained with anti-Flag, anti-CD25, anti-CD54 or control monoclonal antibody conjugated with FITC. After incubation for 30 minutes, the cells were washed and analyzed for the presence of surface antigen in a flow cytometer.

MTT assay

The MTT assay was performed according to the manufacturer's instructions. Briefly, HGFs from seven individuals were seeded at a density of 5×10^3 cells *per* well in a 96-

well plate in triplicate. The tetrazolium compound MTT was added after stimulation for 24 hrs. Detergent reagent was added 2 hrs after addition of MTT and incubated at 37°C for a further 4 hrs. The absorbance was then read at a wavelength of 550 nm.

Statistical analysis

Comparisons between the two groups were performed by the paired Student's t-test. Comparisons between multiple groups were performed by one-way analysis of variance with Tukey's test.

RESULTS

Opposite effects of cyclosporin and phenytoin on TLR activation in CHO reporter cells

To determine the effects of cyclosporin and phenytoin on TLR activation, CHO transfectants were exposed to those drugs. CHO/CD14/TLR2 cells express Flag-tagged TLR2 (Fig. 1A) and are responsive to both TLR2 and TLR4 ligands (Lien *et al*., 1999). CD25 is the reporter molecule for TLR activations, and cyclosporin alone could induce CD25 in CHO/CD14/TLR2 cells. When CHO/CD14/TLR2 cells were stimulated with a TLR4 ligand, ultra pure LPS, or a TLR2 ligand, Pam_3CSK_4, cyclosporin enhanced TLR-mediated activation (Fig. 1B). On the other hand, phenytoin attenuated the TLR-mediated activation (Fig. 1C). To exclude the possibility that these effects of cyclosporin and phenytoin are due to the direct interaction between those drugs and TLR ligands, CHO/CD14/TLR4 cells were treated with different doses of cyclosporin or phenytoin. In this cell line, NF-κB is constitutively activated by overexpression of Flag-tagged TLR4 (Kirschning *et al*, 1998). CD25 expression in CHO/CD14/TLR4 cells was enhanced by cyclosporin but inhibited by phenytoin (Figs. 1D, 1E), indicating that those drugs affected intracellular signaling. These results demonstrated distinctive effects of cyclosporin and phenytoin on TLR-mediated signaling.

Effects of cyclosporin and phenytoin on inflammatory responses in HGFs

Since expression of an adhesion molecule, CD54, can be induced by TLR signaling and is often highly elevated in inflamed periodontal tissues (Gemmell *et al*., 1994), we

investigated the effects of cyclosporin and phenytoin on its surface expression in HGFs.

Cyclosporin and phenytoin alone had only a marginal effect on CD54 expression, but

cyclosporin increased CD54 expression in HGFs stimulated with lipid A or Pam_3CSK_4 in

both serum-containing and serum-free conditions (Figs. 2A, 2B). Phenytoin attenuated

the CD54 expression in serum-containing conditions but had no significant effect in

serum-free conditions (Figs. 2C, 2D).

To investigate the effects of these drugs on *in vivo* CD54 expression, LPS was injected

into the gingiva of mice that were administrated PBS, cyclosporin or phenytoin every 24

hrs for 5 days. The percentage of CD54-positive cells in total fibroblast-like cells in

cyclosporin-treated mice was significantly higher than that in phenytoin-treated mice

(Fig. 2E).

The proinflammatory cytokine IL-6 and the chemokine IL-8 are also important

inflammatory mediators that HGFs produce in response to TLR signaling (Kawai and

Akira, 2006; Mahanonda *et al*., 2006). Similar to CD54 expression, cyclosporin

augmented the production of IL-6 and IL-8 induced by Pam_3CSK_4 in both serum-

containing and serum-free conditions (Figs. 3A, 3B, Appendix). Cyclosporin also

augmented the production of IL-8 induced by lipid A in serum-free conditions but not in

serum-containing conditions, while no significant effect was found on IL-6 release. On

the other hand, phenytoin attenuated the production of IL-6 and IL-8 induced by lipid A

or Pam_3CSK_4 in both serum-containing and serum-free conditions, except for the

production of IL-8 in serum-free conditions (Figs. 3C, 3D, Appendix).

Effects of cyclosporin and phenytoin on growth of HGFs

To evaluate the effects of cyclosporin and phenytoin on cell growth, HGFs were treated

with cyclosporin or phenytoin for 24 hrs and subjected to MTT assay. Both cyclosporin

and phenytoin augmented the growth of HGFs. Treatment of HGFs with lipid A or

Pam_3CSK_4 diminished cell growth, but cyclosporin increased the growth of HGFs

stimulated with those TLR ligands, whereas phenytoin did not exhibit a significant effect

(Fig. 4).

DISCUSSION

It has been reported that cyclosporin enhanced NF-κB activities in human bronchial epithelial cells and the rat kidney (Aoki and Kao, 1997; Asai *et al*., 2003). A more recent study has revealed that inhibition of calcineurin by cyclosporin could trigger TLR signaling in mouse macrophages (Kang *et al*., 2007). In accordance with those findings, our results demonstrated a positive effect of cyclosporin on the activation of CHO reporter cells, although cyclosporin alone could induce only marginal responses of HGFs. Other investigators also reported that cyclosporin alone had no or only a marginal effect on NF-κB translocation (Bostrom *et al*., 2005). The effect of cyclosporin on signal induction in HGFs was not as strong as that in mouse macrophages or CHO cells, but this milder effect seemed to be sufficient to augment the responses of HGFs to TLR ligands.

To our knowledge, there has been no report regarding the effect of phenytoin on TLR signaling. However, lignocaine, which is a voltage-sensitive sodium channel blocker, has been found to inhibit LPS-induced activation of NF-κB and mitogen-activated protein kinases in murine macrophages (Lee *et al*., 2008). Considering that phenytoin has the same pharmacological activity, it might down-regulate the signaling in HGFs in a similar manner. It is noteworthy that the growth of HGFs was increased by treatment with phenytoin as previously reported (Sano *et al*., 2004). This indicated that the inhibitory effect of phenytoin was specific to inflammatory responses and was not due to its toxicity.

In the present study, cyclosporin enhanced IL-6 and IL-8 production and CD54 expression in HGFs stimulated with synthetic TLR ligands. Cyclosporin also enhanced CD54 expression in a mouse *in vivo* model. IL-6 could induce TGF-β production and HGF proliferation (Chae *et al*., 2006). Enhanced production of IL-8 could explain the increased number of microvessels found in cyclosporin-induced gingival overgrowth (Bullon *et al*., 2007) since IL-8 is known to have pro-angiogenic activity (Koch *et al*., 1992). CD54 is important for migration and retention of inflammatory cells in diseased periodontal tissues (Hosokawa *et al*., 2006). The up-regulation of cytokine production was more evident in HGFs stimulated with Pam_3CSK_4 than in those stimulated with lipid A. This might result from the differences in signal transductions. Whereas Pam_3CSK_4 binds TLR2, which in turn induces myeloid differentiation (MyD)88-dependent signaling, lipid A binds TLR4, which induces both MyD88-dependent and TRIF-dependent signaling (Kawai and Akira, 2006).

In contrast to cyclosporin, phenytoin attenuated the cytokine production and CD54 expression in HGFs induced by TLR ligands. These results might be related to lower levels of inflammatory infiltrates observed not only in overgrown gingival tissue (Uzel *et at*., 2001; Trackman *et al*., 2004; Kantarci *et al*., 2007) but also in experimental autoimmune encephalomyelitis after phenytoin treatment (Black *et al*., 2006). In serum-free conditions, the effect of phenytoin on IL-8 production and CD54 expression was not clear. Other investigators have reported that phenytoin alone as well as in combination with IL-1 up-regulated the production of IL-6 and IL-8 in HGFs in serum-free media (Modéer *et al*., 2000). Presumably, culture conditions, such as the presence of serum protein, affect the inhibitory effects of phenytoin.

Even though drug-induced gingival overgrowth is characterized by similar aspects, such as expanded connective tissue and excess collagen fibers (Kataoka *et al*., 2005), accumulating evidence indicates differences in the inflammatory reaction in overgrown gingival tissue depending on the administrated drug (Wondimu *et al*., 1995; Uzel *et al*., 2001; Trackman *et al*., 2004). Our results revealed that cyclosporin positively regulates TLR-mediated inflammatory responses in HGFs and that phenytoin has the opposite effect. The concentrations of cyclosporin and phenytoin used in this study were higher than those found in blood of patients, but consideration should be given to the fact that these drugs are usually taken for long periods and are likely to undergo accumulation in gingival tissue, since dental plaque can act as a reservoir of cyclosporin (McGaw *et al*., 1987). In this aspect, the findings in the present study may explain the distinctive roles of cyclosporin and phenytoin in the inflammatory responses in overgrown gingival tissue. However, it is generally acknowledged that HGFs show considerable interindividual variability. The degree of tissue inflammation associated with gingival overgrowth would be closely related to the HGF capacity for inflammatory responses.

ACKNOWLEDGEMENTS

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LEGENDS

Figure 1

Opposite effects of cyclosporin and phenytoin on TLR signaling in CHO reporter cells.

CHO/CD14, CHO/CD14/TLR2 and CHO/CD14/TLR4 reporter cells were stained with anti-Flag monoclonal antibody conjugated with FITC or isotype-matched control antibody conjugated with FITC and expression of protein tag Flag was analyzed by flow cytometry (A).

CHO/CD14/TLR2 reporter cells remained unstimulated or were stimulated with 30 ng/mL ultra pure LPS or 10 ng/mL of Pam_3CSK_4 in the presence or absence of 30 μg/mL of cyclosporin (B) or 100 μg/mL of phenytoin (C).

CHO/CD14/TLR4 reporter cells were exposed to the indicated concentration of cyclosporin (D) or phenytoin (E). After 18 hrs of incubation, the cells were stained with anti-CD25 monoclonal antibody conjugated with FITC or isotype-matched control antibody conjugated with FITC and analyzed by flow cytometry. Mean values ± SD of the mean fluorescence intensity of each histogram (n =3) are shown. The data are representative of three independent experiments with similar results. ##, $p < 0.01$. P values were calculated in comparison to the controls without lipid A or Pam_3CSK_4 treatment. *, $p < 0.05$, **, $p < 0.01$. P values were calculated in comparison to the controls stained with isotype-matched control antibody (A) or in comparison to the controls with no cyclosporin or phenytoin treatment (B-E).

CsA, cyclosporin; PHT, phenytoin; MFI, mean fluorescence intensity.

Figure 2

  Effects of cyclosporin and phenytoin on TLR-mediated CD54 expression.

HGFs remained unstimulated or were stimulated with 100 ng/mL of lipid A or 500 ng/mL

of Pam_3CSK_4 in the presence or absence of 30 μg/mL of cyclosporin (A, B) or 100

μg/mL of phenytoin (C, D). The experiments were performed either in serum-containing

media (A, C) or serum-free media (B, D). After 18 hrs of incubation, the cells were

stained with anti-CD54 monoclonal antibody conjugated with FITC or isotype-matched

control antibody conjugated with FITC and analyzed by flow cytometry. Mean values ±

SD of the mean fluorescence intensity of each histogram (n =3) are shown. The data

are representative of seven independent experiments with similar results. (E) Mice

administrated PBS, cyclosporin or phenytoin were injected with either PBS (n = 3 in

each group) or LPS (n = 5 in each group) in their gingiva every 24 hrs. After 5 days of

treatment, the gingival tissues were subjected to immunohistochemcal staining of CD54,

and mean ± SD percentages of CD54-positive fibroblast-like cells are shown. #, $p <$

0.05, ##, $p < 0.01$. *P* values were calculated in comparison to the controls without lipid

A or Pam_3CSK_4 treatment. *, $p < 0.05$, **, $p < 0.01$. *P* values were calculated in

comparison to the controls with no cyclosporin or phenytoin treatment (A-D) or in

comparison between drug-treatment groups (E).

CsA, cyclosporin; PHT, phenytoin; MFI, mean fluorescence intensity.

Figure 3

  Effects of cyclosporin and phenytoin on IL-6 production by HGFs.

HGFs remained unstimulated or were stimulated with 10 ng/mL of lipid A or 10 ng/mL

of Pam_3CSK_4 in the presence of 0, 10 or 30 μg/mL of cyclosporin (A, B) or 0, 30 or 100

μg/mL of phenytoin (C, D). The experiments were performed either in serum-containing

media (A, C) or serum-free media (B, D). After 20 hrs of incubation, culture

supernatants were assayed for IL-6 by ELISA. Mean values ± SD (n =3) are shown.

The data are representative of seven independent experiments with similar results. #, *p*

< 0.05, ##, *p* < 0.01. *P* values were calculated in comparison to the controls without

lipid A or Pam_3CSK_4 treatment. *, *p* < 0.05, **, *p* < 0.01. *P* values were calculated in

comparison to the controls with no cyclosporin or phenytoin treatment.

CsA, cyclosporin; PHT, phenytoin.

Figure 4

  Effects of cyclosporin and phenytoin on cell growth.

HGFs remained unstimulated or were stimulated with 100 ng/mL of lipid A or 100 ng/mL

of Pam_3CSK_4 in the presence of 0, 10 or 30 μg/mL of cyclosporin (A) or 0, 30 or 100

μg/mL of phenytoin (B). After 24 hrs of incubation, cell growth was measured by MTT

assay. The data are representative of seven independent experiments with similar

results. ##, *p* < 0.01. *P* values were calculated in comparison to the controls without

lipid A or Pam_3CSK_4 treatment. *, *p* < 0.05; **, *p* < 0.01. *P* values were calculated in

comparison to the controls without cyclosporin or phenytoin treatment.

CsA, cyclosporin; PHT, phenytoin.

Appendix

Effects of cyclosporin and phenytoin on IL-8 production by HGFs.

HGFs remained unstimulated or were stimulated with 10 ng/mL of lipid A or 10 ng/mL

of Pam_3CSK_4 in the presence of 0, 10 or 30 μg/mL of cyclosporin (A, B) or 0, 30 or 100

μg/mL of phenytoin (C, D). The experiments were performed either in serum-containing

media (A, C) or serum-free media (B, D). After 20 hrs of incubation, culture

supernatants were assayed for IL-8 by ELISA. Mean values ± SD (n =3) are shown.

The data are representative of seven independent experiments with similar results. #, p

< 0.05, ##, p < 0.01. P values were calculated in comparison to the controls without

lipid A or Pam_3CSK_4 treatment. *, p < 0.05, **, p < 0.01. P values were calculated in

comparison to the controls with no cyclosporin or phenytoin treatment.
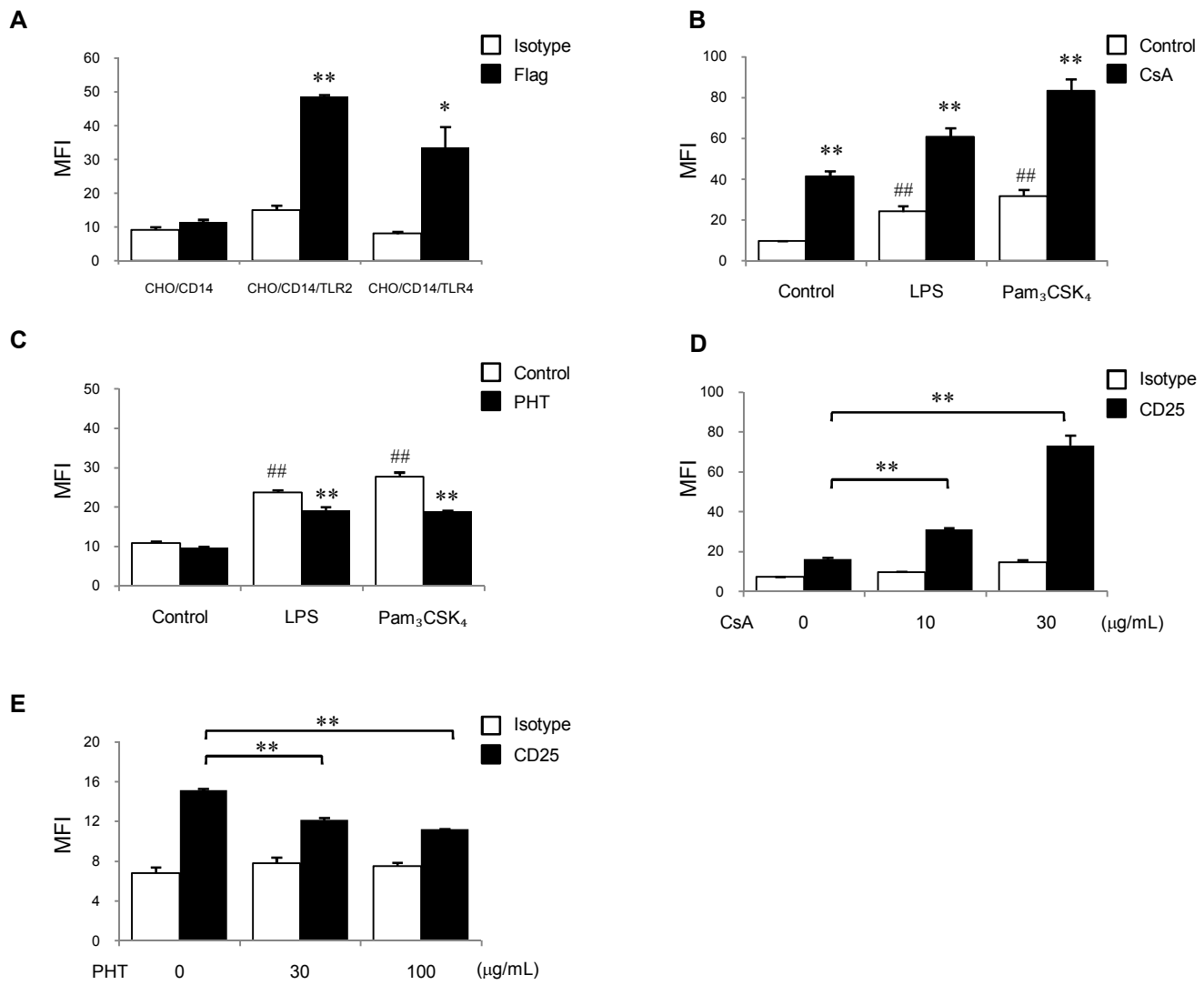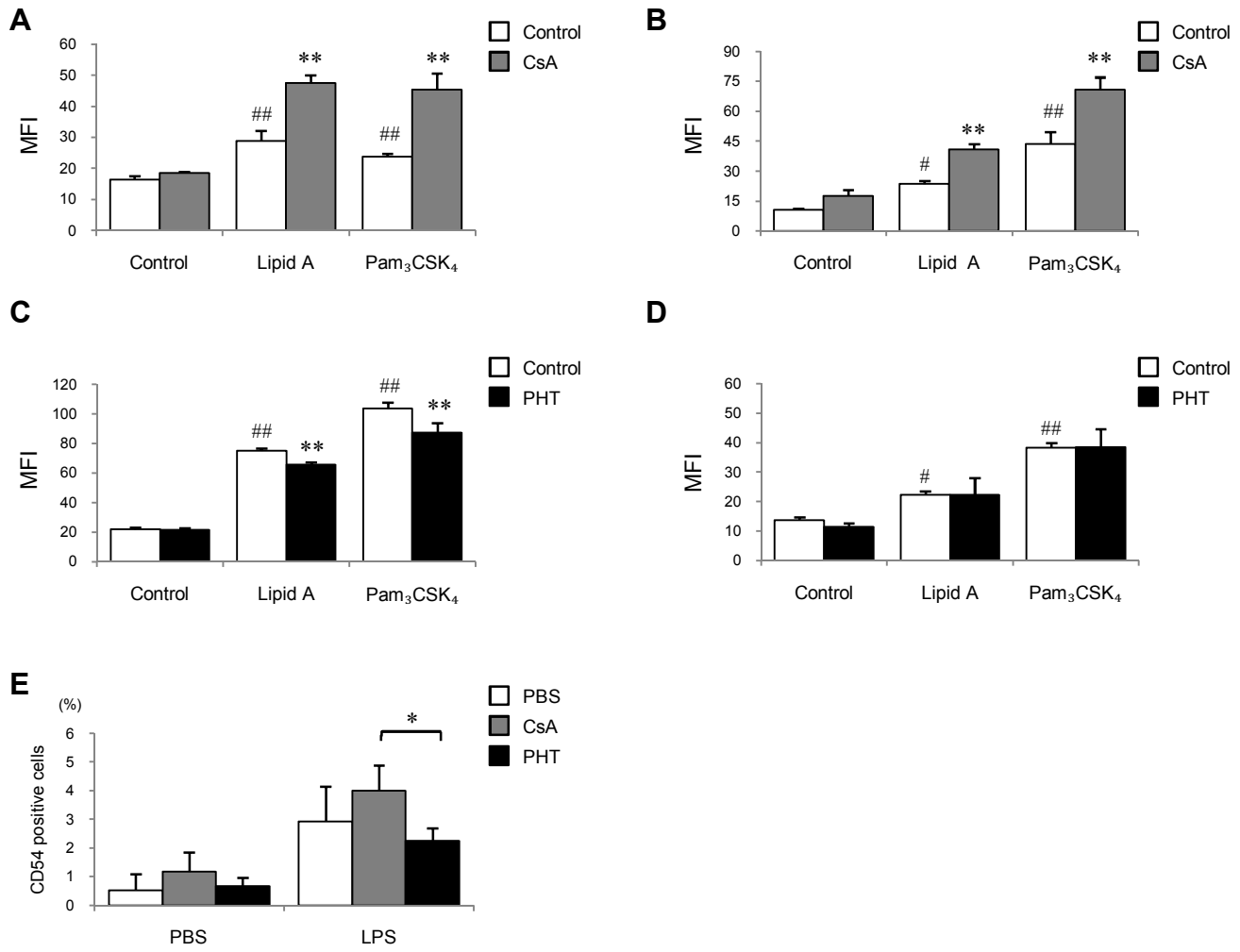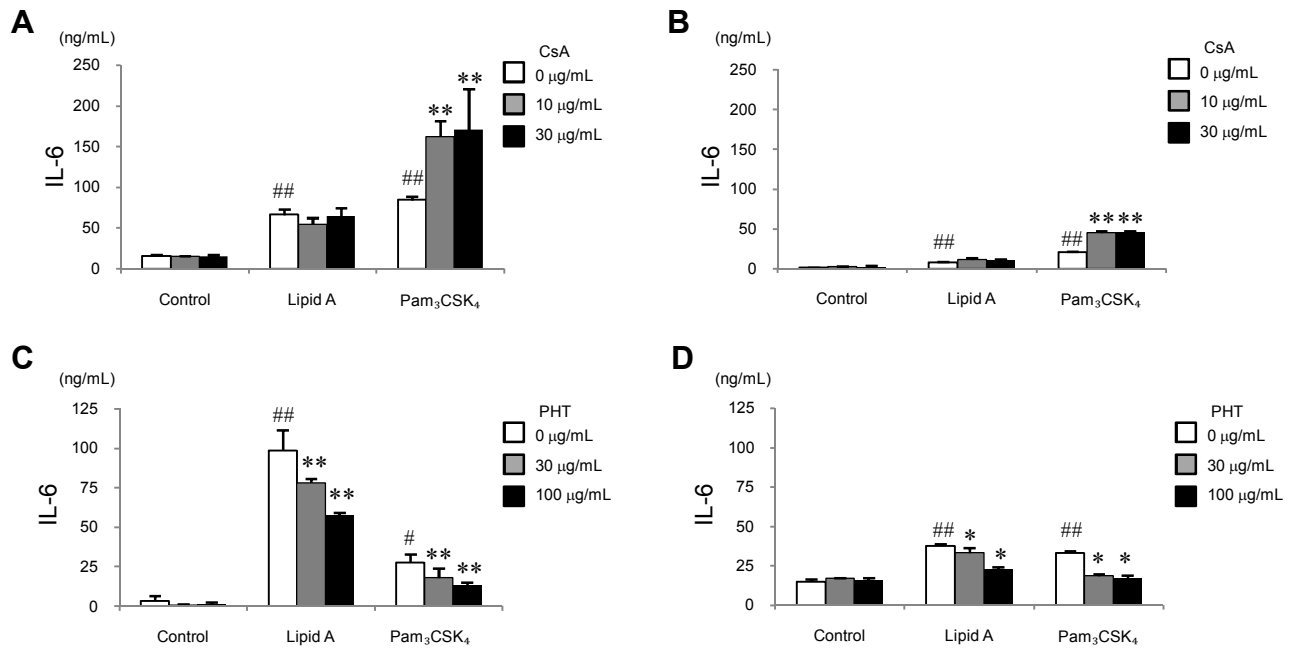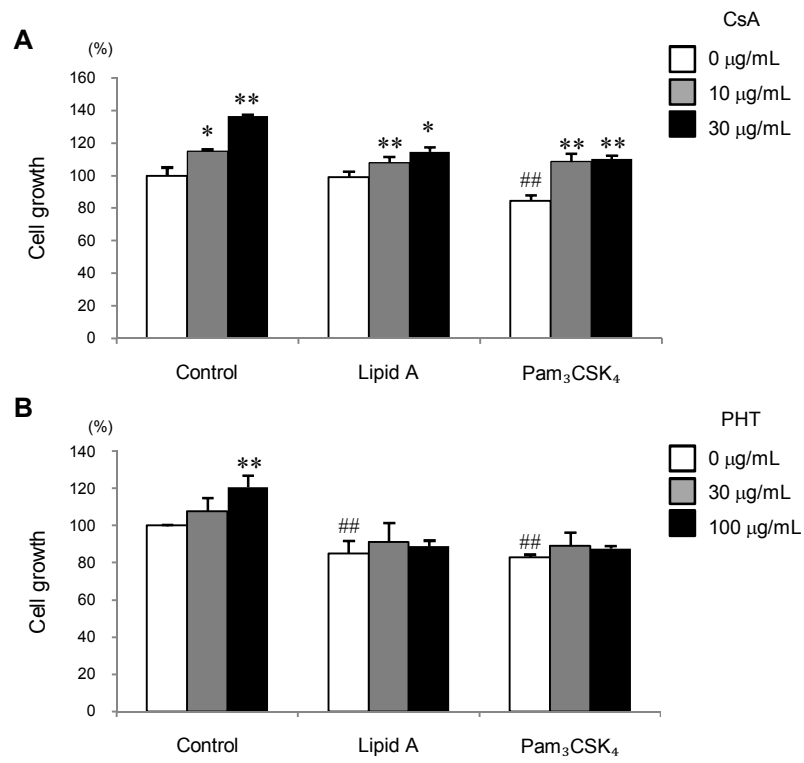
Figure 1

Figure 2

Figure 3

Figure 4

Appendix

A

B

C

D