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Title page

A novel cyclohexene derivative, TAK-242, selectively inhibits Toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling

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Running title: Inhibition of TLR4-mediated cytokine production by TAK-242

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Abbreviations: NO, nitric oxide; iNOS, inducible NO synthase; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-alpha; IL, interleukin; LBP, LPS-binding protein; TLR, Toll-like receptor; PGN, peptidoglycan; LTA, lipoteichoic acid; Poly (I:C),polyinosinic-polycytidylic acid; CpG DNA, non-methylated CpG oligodeoxynucleotide; PBMC, peripheral blood mononuclear cells; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; Erk, extracellular signal-regulated kinase; TAK-242, ethyl (6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate.

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Abstract

Proinflammatory mediators such as cytokines and nitric oxide (NO) play pivotal roles in various inflammatory diseases. To combat inflammatory diseases successfully, regulation of proinflammatory mediator production would be a critical process. In the present study, we investigated the in vitro effects of TAK-242, a novel small molecule cytokine production inhibitor, and its mechanism of action. In RAW264.7 cells and mouse peritoneal macrophages, TAK-242 suppressed lipopolysaccharide (LPS)-induced production of NO, tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-6, with 50% inhibitory concentration (IC_{50}) of 1.1 to 11 nM. TAK-242 also suppressed the production of these cytokines from LPS-stimulated human peripheral blood mononuclear cells (PBMCs) at IC_{50} values of 11 to 33 nM. In addition, the inhibitory effects on the LPS-induced IL-6 and IL-12 production were similar in human PBMCs, monocytes and macrophages. TAK-242 inhibited mRNA expression of IL-6 and TNF-α induced by LPS and interferon-γ in RAW264.7 cells. The phosphorylation of mitogen-activated protein kinases induced by LPS was also inhibited in a concentration-dependent manner. However, TAK-242 did not antagonize the binding of LPS to the cells. Interestingly, TAK-242 suppressed the cytokine production induced by Toll-like receptor (TLR) 4 ligands, but not by ligands for TLR2, 3, and 9. In addition, IL-1β-induced IL-8 production from human PBMCs was not markedly affected by TAK-242. These data

suggest that TAK-242 suppresses the production of multiple cytokines by selectively

inhibiting TLR4 intracellular signaling. Finally, TAK-242 is a novel small molecule TLR4

signaling inhibitor and could be a promising therapeutic agent for inflammatory diseases,

whose pathogenesis involves TLR4.

Introduction

Cytokines and nitric oxide (NO) are involved in a wide variety of inflammatory diseases including sepsis, rheumatoid arthritis (RA), atherosclerosis, inflammatory bowel diseases (IBD), asthma, and chronic obstructive pulmonary disease. In RA, for example, interleukin (IL)-1 is considered to be a mediator. Plasma concentrations of IL-1 in RA patients are elevated and they correlate with disease activity (Eastgate et al., 1988). The expression of IL-1 in the bronchial epithelium of patients with asthma is significantly elevated as compared to healthy volunteers (Sausa et al., 1996). In some animal models of sepsis, hemodynamics and survival are improved when the actions of IL-1 are blocked by an IL-1 receptor antagonist (Fischer et al., 1992; Norman et al., 1995). Tumor necrosis factor-alpha (TNF-α) is also known to exhibit diverse physiologic effects and is one of the most prominent proinflammatory mediators. It can exert host damaging effects in sepsis, fever syndromes and cachexia as well as in autoimmune diseases such as RA, psoriasis, and IBD (Raza, 2000; Hehlgans and Pfeffer, 2005). For example, TNF-α induces the secretion of inflammatory cytokines and chemokines from stroma cells, endothelial cells, and mucosal mononuclear cells in IBD (MacDermott, 1996). NO derived from inducible NO synthase (iNOS) also appears to be a proinflammatory mediator with immunomodulatory effects (Guzik et al., 2003). The toxic properties of NO are the key in the pathogenesis of septic shock and

overproduction of NO during septicemia is considered to be responsible for irreversible

arterial hypotension, vasoplegia, lactic acidosis, necrosis and apoptosis (Parratt, 1997).

Furthermore, the manifestations of allergic airway disease, including infiltration of

eosinophils, microvascular leakage, and the airway occlusion are markedly less severe in

iNOS$^{-/-}$ animals and iNOS promotes allergic inflammation in airways via downregulation of

interferon-gamma (IFN-γ) (Ricciardolo et al., 2004).

Lipopolysaccharide (LPS), a major constituent of Gram-negative bacterial outer membrane,

can cause inflammatory responses such as the release of cytokines and NO from various types

of cells, including monocytes and macrophages. To activate the cells, the lipid A-moiety of

LPS attaches to the LPS-binding protein (LBP), and the LPS/LBP complex binds to CD14 and

is then transferred to the Toll-like receptor (TLR) 4-MD-2 complex (Wright et al., 1990; da

Silva Correia et al., 2001). The resulting activation of the cells induces the activation of

mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB), as well as

release of inflammatory mediators (Guha and Mackman, 2001). TLR4 is also known to be a

receptor for endogenous ligands such as fibrinogen, hyaluronic acid, and heat shock proteins

(Rifkin et al, 2005). Thus, an agent that inhibits TLR4-mediated cytokine and NO production

could be a promising drug for the treatment of inflammatory diseases. In fact, some synthetic

lipid A analogues showed beneficial effects as TLR4 antagonists in septic shock models and

IBD models in mice (Christ et al., 1995; Mullarkey et al., 2003; Fort et al., 2005).

We have discovered a novel cyclohexene derivative, TAK-242, which selectively inhibits

the TLR4-mediated production of cytokines and NO. The chemical structure of TAK-242 is

shown in Fig. 1. TAK-242 is the first small molecule compound that selectively inhibits TLR4

signaling. In this study, we investigated the inhibitory effect of TAK-242 on the production of

inflammatory mediators by macrophages and monocytes as well as its mode of action.

Materials and methods

Materials. TAK-242, Ethyl (6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate was synthesized at Takeda Pharmaceutical Company Limited (Osaka, Japan). AG126 was purchased from Calbiochem (San Diego, CA), LPS (from *Escherichia coli* serotype O111:B4) and lipoteichoic acid (LTA) (from *Staphylococcus aureu*s) from Sigma (St. Louis, MO), and LPS(S) (from *Salmonella typhimurium*) from Difco (Detroit, MI). Recombinant mouse IFN-γ was purchased from Genzyme (Cambridge, MA), while recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) was purchased from PeproTech EC (London, UK). Peptidoglycan from *S. aureus* (PGN) was purchased from Fluka (Buchs, Switzerland), polyinosinic-polycytidylic acid (Poly (I:C)) from Amersham (Piscataway, NJ), paclitaxel (Taxol) and recombinant human IL-1β from Wako (Osaka, Japan), non-methylated CpG oligodeoxynucleotide (CpG DNA) from Hokkaido System Science (Sapporo, Japan), and 2,3-diaminonaphthalene (DAN), which an agent for the detection of nitrite (Misko et al., 1993), from Dojindo Laboratories (Kumamoto, Japan).

Cells. The murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI1640 medium (Nikken Bio Medical Laboratories, Kyoto, Japan) containing 10% heat-inactivated fetal calf serum (FCS) and 10 μg/mL kanamycin at 37°C under a humidified atmosphere with 5% CO_2.

Resident mouse peritoneal macrophages were harvested from BALB/c mice (Charles River Japan, Kanagawa, Japan). The peritoneal cells were seeded at a density of 5×10^5 cells/well in 96-well culture plates (Nunc, Rochester, NY) and cultured in RPMI1640 medium containing 10% heat-inactivated FCS, 100 U/mL penicillin G, and 100 µg/mL streptomycin for 2 h. After shaking the cultures, non-adhesive cells were aspirated. The adhesive cells were washed and used as peritoneal macrophages. Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood obtained from healthy human volunteers by density gradient centrifugation using Ficoll-Paque™ Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and suspended in RPMI1640 medium containing 10% heat-inactivated FCS, 100 U/mL penicillin G and 100 µg/mL streptomycin. To obtain monocytes, PBMCs were washed and suspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA. The cells were then treated with microbeads coated with anti-CD14 monoclonal antibody (Miltenyi Biotec, Gladbach, Germany) and subjected to a magnetic cell separation system according to the manufacturer's protocol. To differentiate the monocytes into macrophages, monocytes obtained using above procedure were plated in 96-well culture plates (Cornig; Corning, NY) at a density of 1×10^4 cells/well and cultured for 7 days in RPMI1640 medium supplemented with 10 ng/mL GM-CSF and 10% FCS at 37°C under a humidified atmosphere with 5% CO_2.

Treatments of the cells. RAW264.7 cells were plated at a density of 1×10^5 cells/well in

96-well culture plates and incubated overnight. After removing cell culture supernatants, the

cells were stimulated with various concentrations of TLR ligands in the presence or absence

of IFN-γ for 20 h in a stimulation medium (phenol red-free RPMI1640 medium containing

1% heat-inactivated FCS and 10 μg/mL kanamycin). Resident mouse peritoneal macrophages

were stimulated with 1 ng/mL LPS and 1 U/mL IFN-γ in RPMI1640 medium containing 10%

heat-inactivated FCS, 100 U/mL penicillin G, and 100 μg/mL streptomycin for 4 h (for

TNF-α and IL-6 assay) or 20 h (for IL-1β and NO assay). For PBMC assay, PBMCs were

seeded at a density of 8×10^4 cells/well in 96-well culture plates and stimulated with 1 ng/mL

LPS and 1 U/mL IFN-γ for 20 h. To examine the efficacy on IL-8 production induced by LPS

and IL-1β, PBMCs were stimulated with 1 ng/mL LPS or 10 ng/mL IL-1β for 20 h. For the

comparison of the efficacy on PBMCs, monocytes and macrophages, the cells were

stimulated with 10 ng/mL LPS in the presence of TAK-242 for 18 h. In another experiment,

human macrophages were stimulated with 10 ng/mL LPS or 20 μg/mL PGN for 18 h. For all

the experiments, TAK-242 was dissolved in N,N-dimethylformamide, diluted with

appropriate medium, and added to the cells just before the stimulation.

Measurement of the concentrations of nitrite and cytokines in the culture

supernatants. Using DAN, the production of NO was estimated by measuring the amount of

nitrite, a stable metabolite of NO, by a fluorometric method (Misko et al., 1993). The

concentration of TNF-α, IL-6, IL-1β, IL-8 and IL-12 in the culture supernatants were

determined by specific enzyme-linked immunosorbent assay [ELISA; Amersham Pharmacia

Biotech UK (Buckinghamshire, UK), R&D Ssystems (Minneapolis, MN), or Genzyme

Techne (Minneapolis, MN)]. Fifty percent inhibitory concentration (IC_{50}) values of TAK-242

were calculated by least-squares linear regression analysis over the descending linear portion

of the log dose-response curve.

Real-time quantitative polymerase chain reaction (PCR) analysis of TNF-α and IL-6

expression. RAW 264.7 cells were seeded at a density of 3 x 10^6 cells/well in 6-well culture

plate (BD Biosciences, Bedford, MA) and incubated overnight. After washing with RPMI

1640 medium supplemented with 1% FCS and 10 µg/mL kanamycin, the cells were

stimulated with 5 ng/mL LPS and 1 U/mL IFN-γ in the presence or absence of TAK-242 (1 -

100 nM) for the indicated time. Culture supernatants were removed and total RNA was

isolated using the total RNA isolation reagent ISOGEN (Nippon Gene, Tokyo, Japan). Total

RNA was reverse transcribed into cDNA by using TaqMan Reverse Transcription Reagents

(Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis of TNF-α and

IL-6 was performed on ABI Prism 7700 (Applied Biosystems) using Pre-Developed TaqMan

Assay Reagents and Universal PCR Master Mix (Applied Biosystems) according to the

manufacturer's instructions. Quantitation of mRNA was performed using the comparative

threshold cycle method. The highest control level attained by the stimulation (without

TAK-242) was regarded as 100%, and the levels of control group at other time points and

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TAK-242-added group were expressed as the percentage of the highest control level.

Western blot analysis. RAW264.7 cells were plated at a density of 5 x 10^5 cells/well in 24-well culture plates and incubated overnight. TAK-242 or tyrphostin AG126, a tyrosine kinase inhibitor, was added to the cells and incubated for 15 min prior to 30-min stimulation with LPS. After the removal of cell culture supernatants, the cells were incubated in lysis buffer [25 mM Tris-HCl (pH7.4), 1 mM EDTA, 100 mM NaCl, 30 mM NaF, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride, 1 μM leupeptine, and 1 mM (4-amidino- phenyl)-methanesulfonyl fluoride] on ice for 10 min. The cell lysates were centrifuged at 15,000 rpm (Himac CF15D2; HITACHI, Ibaragi, Japan) for 10 min. The resultant supernatant was mixed with 1/4 volume of 5 x sodium dodecyl sulfate (SDS) sample buffer (312.5 mM Tris-HCl (pH6.8), 5% SDS, 50% glycerol, 25% 2-mercaptoethanol, and 0.1% bromophenol blue). The proteins in the lysates were separated by SDS-PAGE (10.5 % gel), and blotted onto polyvinylidene difluoride membranes Immunobilon^{TM} (Millipore, Molsheim, France). After blocking the membrane in TBS-T (25 mM Tris-HCl (pH8.0), 150 mM NaCl, 0.05% Tween 20) containing 3% bovine serum albumin, membranes were washed in TBS-T and probed for 1 h with anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) antibody (Ab) (New England Biolabs, Beverly, MA), anti-phospho-p38 MAPK Ab (New England Biolabs), anti-IκBβ (C-20) Ab (Santa Cruz Biotechnology, CA), anti-phospho-c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) Ab (New

England Biolabs) or anti-p67[phox] Ab (BD Biosciences, San Jose, CA). The membranes were washed four times in TBS-T, and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG Fc Ab (Cappel, Aurora, Ohio). After washing the membranes four times in TBS-T, the bands were detected using enhanced luminol reagent (New England Biolabs) according to the manufacturer's instruction.

Assay for LPS-binding to PBMCs. PBMCs were suspended in BSA solution (PBS containing 0.1% BSA and 0.01 % sodium azide). In a total volume of 50 μL, PBMCs (3 x 10^5 cells) were incubated with TAK-242, anti-human CD14 monoclonal antibody (MAb) MEM-18 (MONOSAN, Uden, The Netherlands), or anti-human CC chemokine receptor 5 (CCR5) MAb (2D7; Pharmingen, San Diego, CA) as a negative control for 30 min at 4°C. The cells were further incubated with 50 ng/mL of LPS from *E. coli* serotype O55:B5 conjugated with Alexa Fluor® 488 (Molecular Probes, Eugene, OR) per mL in the presence of human serum at a final concentration of 1% for 45 min at 37°C. After washing twice with BSA solution, 1 x 10^4 cells were analyzed by flow cytometry using CytoACE300 cytofluorometer (Jasco Corporation, Tokyo, Japan). The assays were performed in triplicate for each PBMCs prepared from four different donors. Specific LPS binding was estimated by subtracting the percentage of LPS-binding cells in the absence of LPS from that in the presence of LPS.

Results

Inhibitory effect of TAK-242 on the production of inflammatory cytokines and nitric oxide from LPS-stimulated monocytes, macrophages, and PBMCs. Resident mouse peritoneal macrophages were stimulated with 1 ng/mL LPS and 1 U/mL IFN-γ in the presence of various concentrations of TAK-242, and the amounts of nitrite (a stable metabolite of NO), TNF-α, IL-6, and IL-1β produced in the supernatants were measured. TAK-242 inhibited the production of these proinflammatory mediators in a concentration-dependent manner, with IC_{50} values ranging from 5.7 to 11 nM (Table 1, Fig. 2). TAK-242 also suppressed the production of NO, TNF-α, and IL-6 from RAW264.7 cells stimulated with 5 ng/mL LPS and 1 U/mL IFN-γ with IC_{50} values ranging from 1.1 to 3.9 nM. In addition, TAK-242 showed similar suppressive effects on the porinflammatory mediator production when RAW264.7 cells were stimulated with a high concentration (1 μg/mL) of LPS alone. NO production from RAW264.7 cells induced by IFN-γ alone was partially suppressed only by more than several hundreds times higher concentrations of TAK-242 compared to those for suppressing the LPS-induced activation (data not shown). TAK-242 didn't show cytotoxicity at a concentration of 10 μM by using the thiazolyl blue tetrazolium bromide method (data not shown).

TAK-242 was also effective in human cells and inhibited the production of TNF-α, IL-6

and IL-1β from PBMCs stimulated with 1 ng/mL LPS and 1 U/mL IFN-γ, with IC$_{50}$ values of

TAK-242 ranging from 5.3 to 58 nM (Table 2, Fig. 3). No marked difference in the IC$_{50}$

values of TAK-242 was observed among PBMCs derived from four different donors. The

efficacy of TAK-242 in human PBMCs was similar to but slightly lower than that in the

resident mouse peritoneal macrophages under the same stimulation condition. As shown in

Table 3, TAK-242 also inhibited the LPS-induced IL-12 production, with IC$_{50}$ values similar

to those for IL-6. Furthermore, it should be noted that TAK-242 showed similar effects on

human PBMCs, monocytes, and GM/CSF-differentiated macrophages. These results suggest

that TAK-242 could show suppressive effects on the production of various inflammatory

mediators from both mouse and human monocytes and macrophages stimulated with LPS.

Inhibitory effect on mRNA expression in RAW264.7 cells. To determine whether the

suppressive effect of TAK-242 on the cytokine production occurs at mRNA expression level,

we used quantitative real-time PCR to examine IL-6 and TNF-α mRNA expressions in

RAW264.7 cells stimulated with LPS and IFN-γ. As shown in Fig. 4, IL-6 mRNA expression

was detected at 2 h after the stimulation, and the level of expression increased thereafter. On

the other hand, TNF-α mRNA expression increased rapidly and reached a maximum level at

1 h after the stimulation with LPS and IFN-γ. These increases in TNF-α and IL-6 mRNA

expression levels were clearly suppressed by TAK-242 at concentrations of 10 to 100 nM

(Fig. 4), indicating that TAK-242 suppresses the production of cytokines by inhibiting the

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mRNA expression.

Inhibitory effect on the LPS-induced activation of MAPK cascades and IκB degradation in RAW264.7 cells. LPS activates various intracellular signaling cascades such as MAPK pathway and NF-κB pathway in monocytes and macrophages, which are required for the induction of many cytokines (Guha and Mackman, 2001). Therefore, we next examined the effect of TAK-242 on the LPS-induced phosphorylation of MAPKs and IκB degradation in RAW264.7 cells. TAK-242 markedly inhibited the LPS-induced phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2), p38 and JNK/SAPK as well as degradation of IκBβ at a concentration of 100 nM. (Fig. 5). Tyrosine kinase inhibitor AG-126 also inhibited the LPS-induced phosphorylation of Erk1/2 and IκBβ degradation; however, it did not inhibit p38 phosphorylation.

Effect of TAK-242 on LPS-binding to PBMCs. The results described above suggest that TAK-242 might target an upstream event in LPS signaling or inhibit LPS binding to the cells. It is known that LPS binds to CD14/TLR4/MD-2 complex on host cells such as monocytes and macrophages (Wright et al., 1990; da Silva Correia et al., 2001; Guha and Mackman, 2001). We conducted experiments to evaluate the effect of TAK-242 on LPS binding to the cells. Human PBMCs were used in this experiment to use a neutralizing anti-human CD14 MAb as a positive control in this experiment. The cells were incubated with fluorescein-conjugated LPS and the LPS binding was analyzed by flow cytometry.

Preincubation of PBMCs with anti-CD14 MAb resulted in complete inhibition of the binding

of LPS to PBMCs; however, the binding was not blocked by anti-CCR5 MAb. Thus, the

binding of LPS to PBMCs was CD14-dependent. In contrast to the anti-CD14 MAb,

TAK-242 did not block the binding of LPS to PBMCs even at a concentration of 10 μM

(Fig.6). However, TAK-242 at a concentration of 1 μM inhibited the production of TNF-α

and IL-6 from PBMCs stimulated under conditions similar to those of the LPS binding assay

(50 ng/mL LPS) by more than 85% when compared with that in the absence of TAK-242

(data not shown). These results suggest that TAK-242 inhibits cytokine production without

antagonizing the binding of LPS to CD14/TLR4/MD-2 complex.

Selective inhibitory effect on TLR4-mediated signaling pathway. We investigated

whether the inhibitory effect of TAK-242 is specific for LPS-induced responses. A lot of

studies have revealed that TLRs are the key molecules for recognizing pathogen-associated

molecular patterns (PAMPs) in order to elicit inflammatory responses and LPS is a

well-known TLR4 ligand. Therefore, RAW264.7 cells were stimulated with various TLR

ligands, and the effect of TAK-242 on cytokine production was examined. In addition to LPS

from *E. coli*, we used LPS (S) (LPS from *S. typhimurium*), LTA and paclitaxel (a diterpene

from a plant) as TLR4 ligands (Takeuchi et al., 1999; Byrd-Leifer, 2001). PGN, Poly (I:C)

and CpG DNA were used as ligands for TLR2, 3, and 9, respectively (Takeuchi et al., 1999;

Alexopoulou et al., 2001; Hemmi et al., 2000). As shown in Fig. 7, TAK-242 inhibited TNF-α

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production from RAW264.7 cells stimulated with LPS (S), LTA and paclitaxel in a concentration-dependent manner similar to LPS *from E.coli*. In contrast, TAK-242 did not show an inhibitory effect on the TNF-α production induced by PGN, Poly (I:C), and CpG. TNF-α production induced by a cell permeable ceramide C2 was not also inhibited by TAK-242 (data not shown). Additionally, similar selective inhibitory patterns were observed in IL-6 and NO production (data not shown). We confirmed the selective inhibitory effect of TAK-242 on TLR4-mediated cytokine production in human macrophages. TAK-242 inhibited IL-6 and IL-12 production in human macrophages stimulated with LPS, with IC_{50} values of 32 and 16 nM, respectively. In contrast, TAK-242 did not inhibit IL-6 and IL-12 production induced by PGN even at a concentration of 2500 nM (Table 4). Furthermore, TAK-242 markedly inhibited IL-8 production from PBMCs induced by LPS, but showed only a marginal inhibitory effect on IL-1β-induced IL-8 production at higher concentrations (Fig. 8). These results suggest that TAK-242 selectively inhibits cytokine production mediated by TLR4, but not by TLR2, 3, 9 or IL-1β.

Discussion

In this paper, we have presented a novel small molecule cytokine production inhibitor, TAK-242 (Fig. 1), which selectively suppresses TLR4-mediated production of cytokines and NO from monocytes and macrophages. Some synthetic lipid A analogues have been reported as LPS antagonists or TLR4 antagonists (Christ et al., 1995; Rossignol and Lynn, 2002; Fort et al., 2005). However, TAK-242 is the first small molecule compound that selectively suppresses TLR4-mediated cytokine production. TAK-242 suppressed the LPS-induced production of TNF-α, IL-1β, IL-12, and NO at similar concentrations (Tables 1, 2, Figs. 2, 3). These data suggest that TAK-242 could show suppressive effects on the production of various types of inflammatory mediators, including those examined in this study. In addition, the inhibitory effects of TAK-242 on cytokine production were similar both in mouse and human macrophages; this suggests that differences in species do not greatly affect the efficacy of TAK-242. The LPS plus IFN-γ-induced increase in mRNA expression levels of IL-6 and TNF-α was also suppressed by TAK-242 at similar concentrations (Fig. 4). These observations have led us to speculate that TAK-242 targets an event that is elicited earlier than the transcription of cytokine genes. Therefore, we examined the effect of TAK-242 on MAPK and NF-κB signaling pathways. TAK-242 inhibited the LPS-induced phosphorylation of Erk1/2, p38 and JNK/SAPK as well as IκB degradation in RAW264.7 cells to a similar

extent (Fig. 5). Although we didn't address the effect of TAK-242 on the direct NF-κB

activation, it is suggested that TAK-242 might inhibit the early process of LPS signaling

upstream of the phosphorylation of MAPKs and the IκB degradation.

The initial process in the activation of immune cells by LPS is the recognition of LPS by a

receptor complex composed of CD14, TLR4, and MD-2 on the cell surface (da Silva Correia

et al., 2001). However, it has been reported that LPS binding to the complex is two types,

namely, LBP/CD14-dependent and -independent types. The binding of LPS to the cells is

LBP/CD14-dependent for LPS concentrations up to 100 ng/mL, and at higher LPS

concentrations, the binding of LPS is LBP/CD14-independent (Triantafilou et al., 2000). To

determine whether TAK-242 inhibits cytokine production through both these types, we used

two stimulation conditions with different concentrations of LPS, i.e., 5 ng/mL (plus IFN-γ)

and 1 μg/mL. Regardless of the LPS concentrations, TAK-242 showed similar suppressive

effects on the production of these mediators from RAW264.7 cells (Table 1). Furthermore,

TAK-242 did not block the CD14-mediated binding of LPS to PBMCs although it suppressed

the cytokine production (Fig. 6). Taken together, TAK-242 is not an LPS antagonist but can

inhibit an LPS-induced signaling process that is elicited after binding of LPS to the receptor

complex.

LPS as well as other microbial components initiate signal transduction through TLRs,

resulting in the release of inflammatory cytokines. TLRs are broadly distributed on the cells

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of the immune system (Muzio and Mantovani, 2000) and recognize a remarkably diverse

array of bacterial, viral, and fungal molecular patterns (Hopkins and Sriskandan, 2005). For

example, it is well known that TLR2, 3, 4 and 9 recognize PGN, Poly (I:C), LPS, and CpG

DNA, respectively (Takeuchi et al., 1999; Alexopoulou et al., 2001; Hemmi et al., 2000).

Interestingly, experiments employing cell stimulation with various ligands for TLR/IL-1

receptor family showed that TAK-242 selectively suppressed TLR4-mediated cytokine

production. TAK-242 inhibited cytokine production in RAW264.7 cells stimulated with

TLR4 ligands; however, it did not show inhibitory effects on ligands for TLR2, 3 or 9 (Fig.

7). In addition, the TLR4-selective inhibition was also observed in human PBMCs and

macrophages (Table 4, Fig. 8). Further, TAK-242 showed similar inhibitory effects on

cytokine production from RAW264.7 cells stimulated with not only LPS from *E. coli* but also

LPS from S. *typhimurium*, LTA *from S. aureus* and paclitaxel (Fig. 7). It should be noted that

we used commercial LTA from *S. aureus* as a TLR4 ligand, as reported previously (Takeuchi,

1999). Although it has been reported that highly purified LTA is a TLR2 ligand (Ellingsen et

al., 2002), cytokine production induced by cell walls derived from *S. aureus* is partially

abolished in TLR4-deficient macrophages (Takeuchi et al., 1999). It is plausible that

TAK-242 inhibits the cytokine production induced by an unknown active TLR4 ligand

contaminated in commercial LTA. Thus, these data suggest that TAK-242 does not

discriminate between TLR4 ligands with regard to the structural differences and could

suppress the activation of cells by a wide range of TLR4 ligands. Further investigation on its

precise mechanism of action is needed along with the elucidation of target molecules of

TAK-242. However, based on the data reported in this study, it can be inferred that TAK-242

might target an upstream event in TLR4-mediated signaling.

Intracellular signaling of TLRs is elicited from Toll/IL-1 receptor (TIR) domain, which is

conserved among the cytoplasmic regions of TLRs. Following the exposure of the cells to

LPS, TLR4 homodimerizes and recruits four adaptor molecules that contain TIR domain, ,

namely, MyD88, MyD88 adaptor-like (Mal, also known as TIRAP), TIR domain-containing

adaptor molecule-1(TICAM-1, also known as TRIF) and TICAM-2 (also known as TRAM)

(Dunne and O'Neill, 2005). Two signaling pathways have been suggested downstream of

TLR4, namely, MyD88-dependent and MyD88-independent pathways. MyD88-deficient

mice did not show production of inflammatory cytokines induced by various TLR ligands.

Mal/TIRAP has been shown to be essential for the MyD88-dependent signaling pathway via

TLR2 and TLR4. TICAM-1/TRIF has been demonstrated to be essential for TLR3- and

TLR4-mediated MyD88-independent pathways (Yamamoto et al., 2002; Oshiumi et al.,

2003a; Fitzgerald et al., 2003). Among the four adaptors, TICAM-2/TRAM specifically

interacts with TLR4 and is involved in a TLR4-mediated signaling pathway (Yamamoto et al,

2003; Oshiumi et al., 2003b, Fitzgerald et al., 2003). TICAM-2/TRAM-deficient mice show

defects in cytokine production in response to TLR4 ligand, but not to other TLR ligands.

MD-2 is also a TLR4-specific molecule; it is a co-receptor of TLR4, which is essential for

LPS signaling of TLR4 (Shimazu et al, 1999; Nagai et al., 2002). MD-2 is physically

associated with the extracellular domain of TLR4 and augments TLR4-dependent LPS

responses. In MD-2 deficient embryonic fibroblasts, TLR4 does not reach to the plasma

membrane and predominantly resides in the Golgi apparatus; this suggests that MD-2 is also

essential for appropriate intracellular distribution of TLR4. Although the target molecule of

TAK-242 remained to be identified, TLR4 and its associated molecules, MD-2, CD14, LBP

and TICAM-2/TRAM may be involved in its inhibitory mechanism. Among these, TLR4,

MD-2 and TRAM might be the most probable candidates for the target because TAK-242 is a

selective inhibitor for TLR4-mediated cytokine production. TAK-242 might directly inhibit

TLR4, TRAM or MD-2. Otherwise, TAK-242 might suppress or activate an unknown

molecule that is uniquely required to regulate TLR4 signaling.

Some TLRs play an important role in the pathogenesis of infectious and inflammatory

diseases such as sepsis, meningitis, atherosclerosis, inflammatory bowel disease, hepatitis,

and autoimmune diseases, e.g., multiple sclerosis and systemic lupus erythematosus (O'Neill,

2003). The involvement of TLR4 in some diseases such as sepsis and atherosclerosis was

indicated based on studies on polymorphisms in the TLR4 gene (Lorenz et al., 2002; Kiechl et

al., 2002). In addition, because tissue macrophages play an important role in the pathogenesis

of various inflammatory diseases (Linton and Fazio, 2003; Schwacha, 2003), it is essential

that drugs used to treat those diseases should act effectively on macrophages as well as on

monocytes. The efficacy of TAK-242 against the LPS-induced IL-6 and IL-12 production was

almost the same between human monocytes and GM-CSF-differentiated macrophages (Table

3). Thus, TAK-242 could offer a new therapeutic approach for inflammatory diseases whose

pathogenesis involves TLR4. In fact, TAK-242 protected mice when tested in the endotoxin

shock model and showed beneficial effects in some sepsis models (in preparation for

publication). Based on the beneficial effects observed in preclinical studies, a clinical trial of

TAK-242 in severe sepsis is now ongoing.

In conclusion, we discovered a novel cyclohexene derivative, TAK-242, which selectively

suppresses TLR4-mediated cytokine production. TAK-242 could be a promising drug for the

treatment of inflammatory diseases involving TLR4, such as sepsis. The precise mechanism

of action of TAK-242 is being investigated.

Acknowledgements

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Legends for figures

Fig. 1. Structure of TAK-242.

Fig. 2. Inhibitory effect of TAK-242 on NO and cytokine production from mouse peritoneal macrophages. The cells were stimulated with LPS (1 ng/mL) and IFN-γ (1 U/mL) in the presence or absence of TAK-242, and the levels of nitrite and cytokines in culture supernatants were measured as described in Materials and Methods. The data are expressed as mean $\pm$ SE (N=3).

Fig. 3. Inhibitory effect of TAK-242 on IL-1β, TNF-α and IL-6 production from human PBMCs. The cells were stimulated with LPS (1 ng/mL) and IFN-γ (1 U/mL) in the presence or absence of TAK-242, and the levels of nitrite and cytokines in culture supernatants were measured as described in Materials and Methods. The data are expressed as mean $\pm$ SE of percent inhibition for PBMCs prepared from four different donors. The levels of IL-1β, TNF-α and IL-6 produced by PBMCs of each donor were 12-38 pg/mL, 34-120 pg/mL, and 450-740 pg/mL, respectively.

Fig. 4. Inhibitory effect of TAK-242 on mRNA expression induced by LPS and IFN-γ in

RAW264.7 macrophages. Cells were incubated with LPS (5 ng/mL) and IFN-γ (1 U/mL) in the presence or absence of TAK-242. Expression levels of TNF-α and IL-6 mRNA were determined by real-time quantitative PCR, and expressed as the percentage of the highest control level (the expression level at 1 h for TNF-α and at 4 h for IL-6). The data are expressed as mean ± SE (N=3).

Fig. 5. Inhibitory effect of TAK-242 on the LPS-induced phosphorylation of MAPKs and IκB degradation in RAW264.7 macrophages. Cells were treated with the indicated concentrations of TAK-242 or tyrphostin AG126 for 15 min prior to stimulation. After the LPS stimulation for 30 min, whole-cell lysates were prepared and subjected to western blot analysis using antibodies specific for phospho-Erk1/2, phospho-p38, phospho- JNK/SAPK, IκBβ, or p67^{phox}. p67^{phox} was used as control.

Fig. 6. Effect of TAK-242 on LPS-binding to PBMCs in the presence of human serum. PBMCs were incubated with anti-human CD14 MAb MEM-18, anti-human CCR5 MAb, or TAK-242 for 30 min at 4 °C and further incubated with 50 ng/mL Alexa-Fluor® 488 conjugated LPS in the presence of 1% human serum for 45 min at 37 °C. The cells were analyzed by flow cytometry. The data are expressed as mean ± SE of four independent experiments using PBMCs prepared from four different donors.

Fig. 7. Ligand specificity for the inhibitory effect of TAK-242 on TNF-α production from RAW264.7 macrophages. Cells were plated at a density of 1 x 10^5 cells/well in 96-well culture plates and incubated overnight. After removing the cell culture supernatants, the cells were stimulated with various TLR ligands described in the presence or absence of TAK-242 for 20 h. The concentration of TNF-α in the culture supernatant was determined in duplicate by specific ELISA.

Fig. 8. Effect of TAK-242 on IL-8 production from human PBMCs stimulated with LPS or IL-1β. The cells were stimulated with IL-1β (10 ng/mL) or LPS (1 ng/mL) in the presence or absence of TAK-242, and IL-8 levels in culture supernatants were measured as described in Materials and Methods. The data are expressed as mean ± SE of percent inhibition for PBMCs prepared from four different donors. The IL-8 levels produced by PBMCs of each donor were 0.85-1.6 ng/mL and 7.7-14 ng/mL for IL-1β and LPS stimulation, respectively.

Tables

TABLE 1

IC_{50} values of TAK-242 for NO, TNF-α, IL-6 and IL-1β production by mouse macrophages

		IC_{50} (nM)			
		(95% confidence intervals)			
Cells	Stimuli	NO	TNF-α	IL-6	IL-1β
Peritoneal macrophages	LPS + IFN-γ [a]	7.7	8.7	11	5.7
		(3.6-34)	(5.2-16)	(8.2-16)	(4.2-8.1)
RAW264.7 cells	LPS + IFN-γ [b]	3.8	3.9	1.1	N.D.
		(2.7-5.3)	(2.5-7.6)	(0.79-1.6)	
	LPS [c]	3.9	4.6	3.7	N.D.
		(1.1-9.8)	(1.9-10)	(2.1-6.0)	

N.D., not determined.

[a] 1 ng/mL LPS and 1 U/mL IFN-γ.

[b] 5 ng/mL LPS and 1 U/mL IFN-γ.

[c] 1 μg/mL LPS.

TABLE 2

IC_{50} values of TAK-242 for inhibition of IL-1β, TNF-α and IL-6 production by human

PBMCs

Donor	IC_{50} (nM)		
	IL-1β	TNF-α	IL-6
A	10	6.0	28
B	21	11	37
C	26	20	11
D	14	5.3	58
Mean ± SE	18 ± 3.5	11 ± 3.3	33 ± 9.8

The 95% confidence intervals of the IC_{50} values have not been shown because the variance of

the duplicate data in low concentrations of TAK-242 was too large for precise estimation.

TABLE 3

IC_{50} values of TAK-242 for inhibition of IL-6 and IL-12 production by human PBMCs, monocytes, and macrophages

Cells	IC_{50} (nM) (95% confidence interval)	
	IL-6	IL-12
PBMCs	330	98
	- [a]	(62-160)
Monocytes	130	56
	(71-270)	(22-100)
Macrophages	76	32
	(37-190)	(23-47)

[a]The 95% confidence interval has not been shown because the variance of duplicate data in low concentrations of TAK-242 was too large for precise estimation.

TABLE 4

IC$_{50}$ values of TAK-242 for lipopolysaccharide (LPS)- and peptidoglycan (PGN)-induced

IL-6 and IL-12 production by human macrophages

	IC$_{50}$ (nM)	
	(95% confidence interval)	
Stimuli	IL-6	IL-12
LPS	32	16
	(19-67)	(6.2-36)
PGN	>2500	>2500

Figure 1
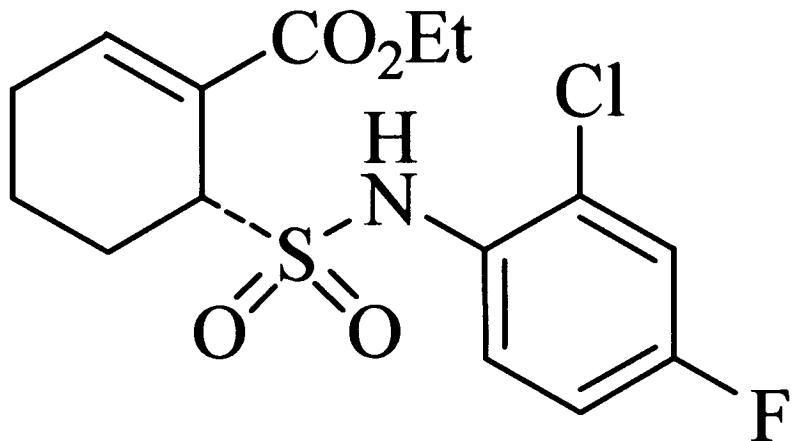
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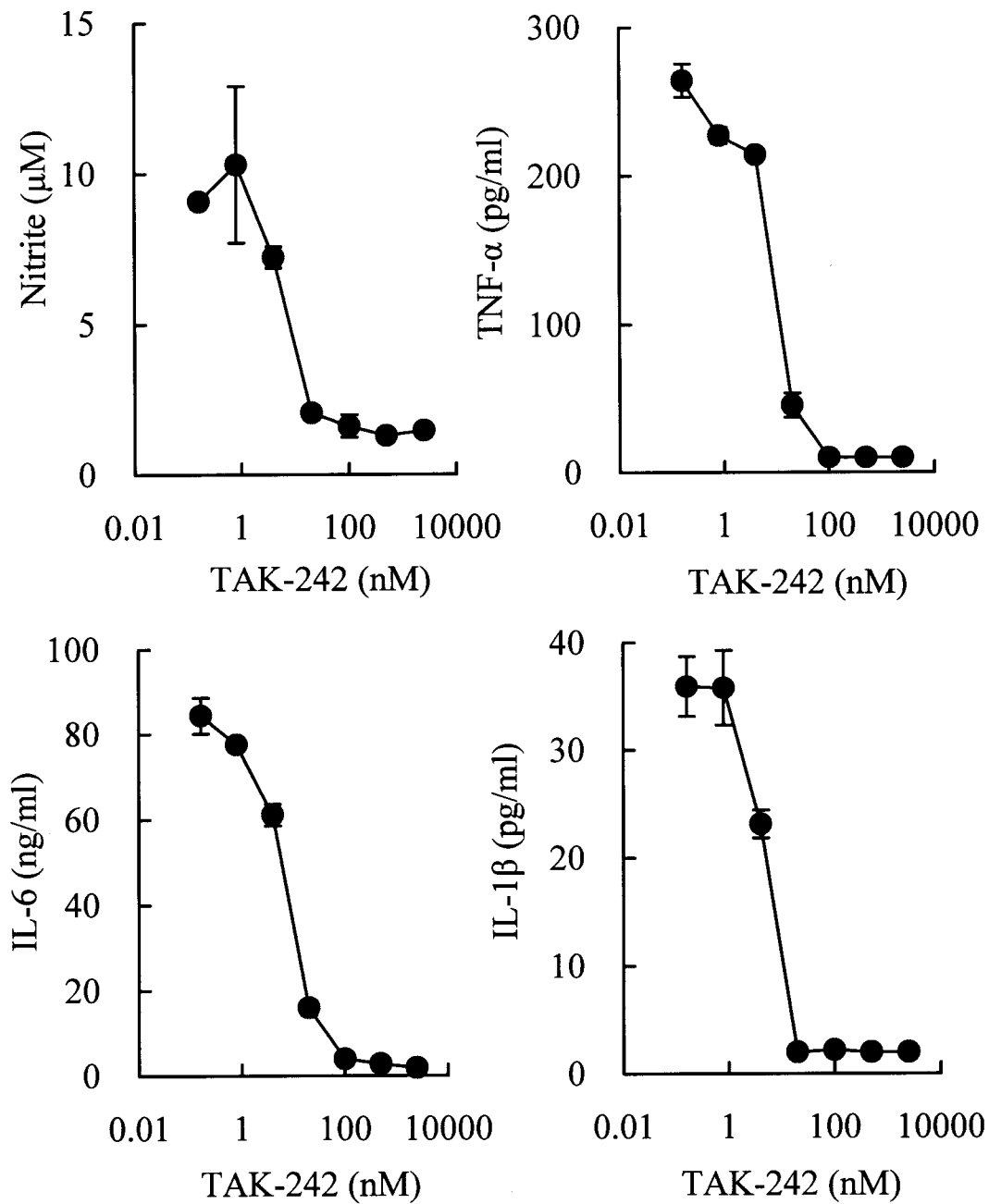
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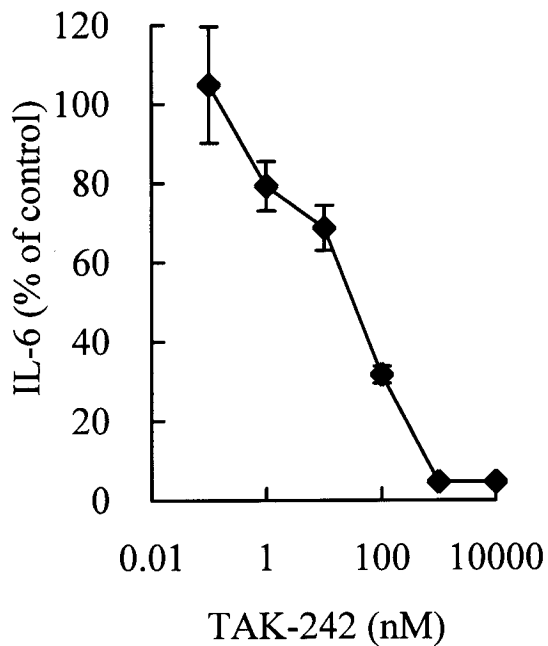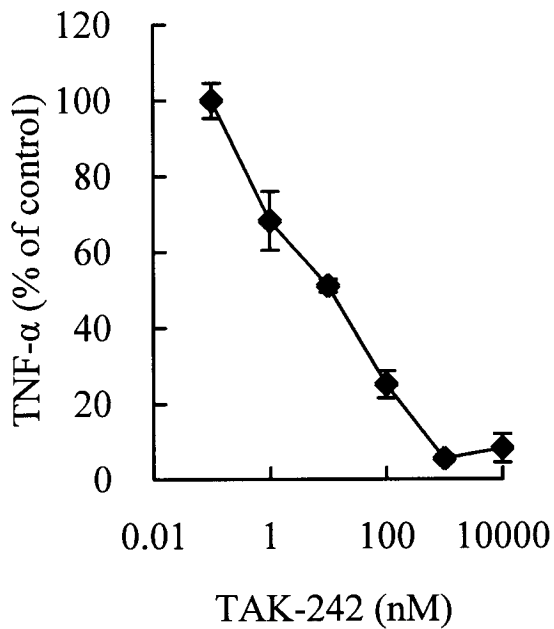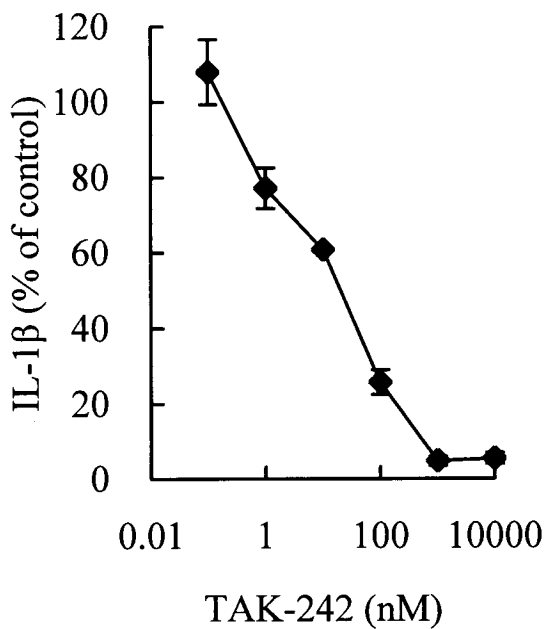
Figure 4

Figure 5

							TAK-242											AG126				
Inhibitor -log(M)	-	-	-	-	-	-	6	6	6	6	6	6	-	9	8	7	6	5	-	7	6	5	4
LPS -log(g/mL)	-	10	9	8	7	6	-	10	9	8	7	6	8	8	8	8	8	8	8	8	8	8	8

phospho-Erk1/2

phospho-p38

phospho-JNK/SAPK
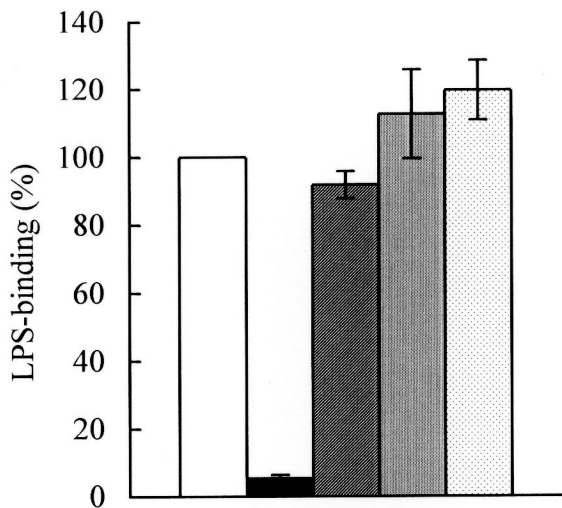
IκBβ

p67^{phox}
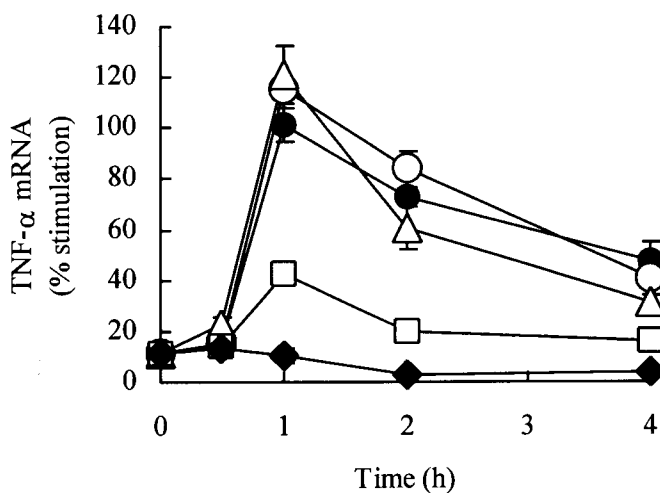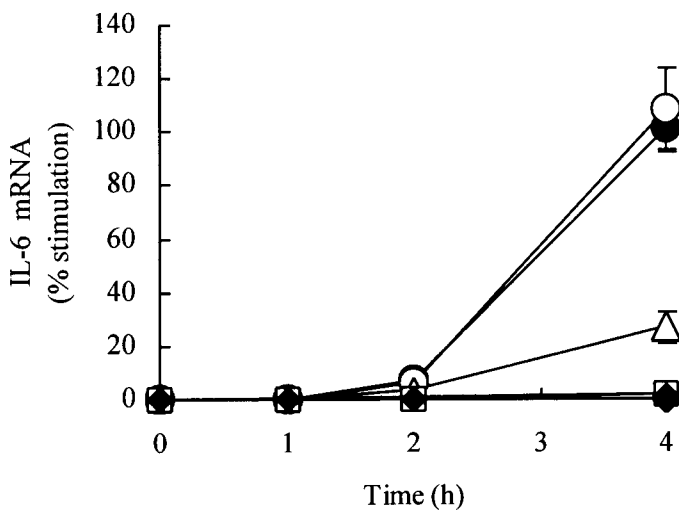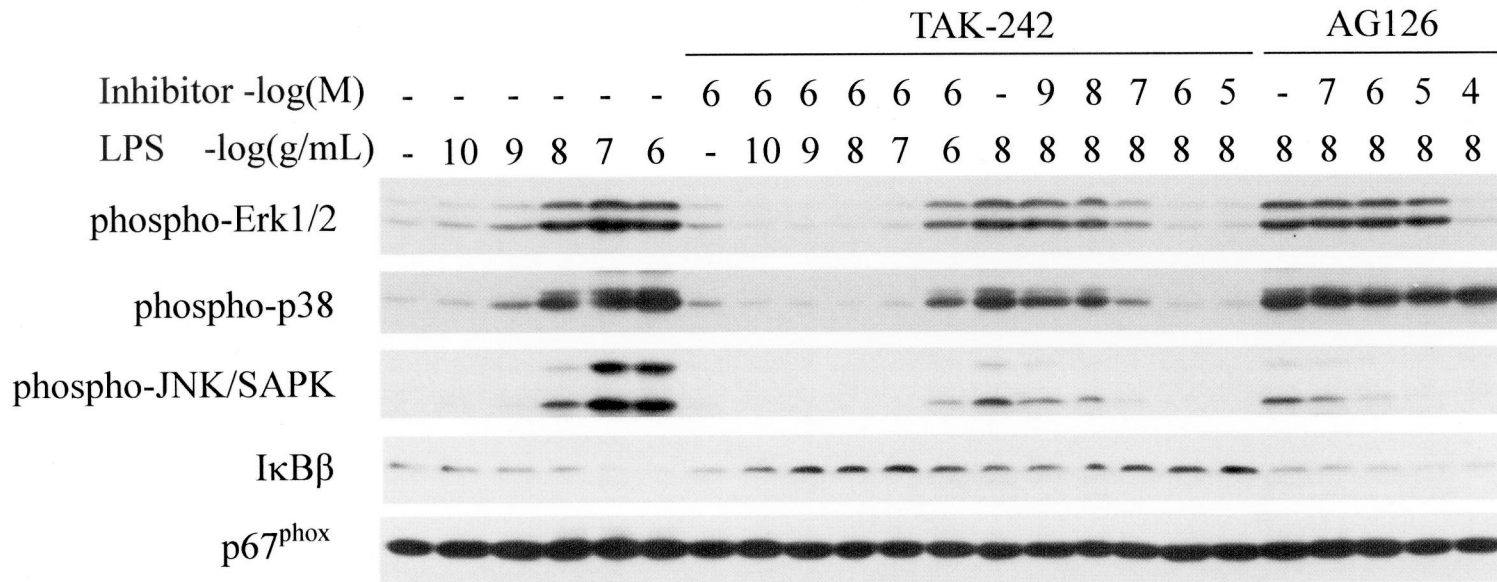
Figure 6

- □ LPS
- ■ LPS + anti-CD14 MAb
- ▨ LPS + anti-CCR5 MAb
- ▩ LPS + TAK-242 1 μM
- □ LPS + TAK-242 10 μM

Figure 7

Figure 8