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European Journal of Pharmacology 584 (2008) 40–48
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TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain

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Received 6 September 2007; received in revised form 27 December 2007; accepted 22 January 2008
Available online 5 February 2008

Abstract

TAK-242, a small-molecule antisepsis agent, has shown to suppress lipopolysaccharide (LPS)-induced inflammation. In this study, we demonstrate that TAK-242 is a selective inhibitor of Toll-like receptor (TLR)-4 signaling. TAK-242 almost completely suppressed production of nitric oxide (NO) or tumor necrosis factor (TNF)-α induced by a TLR4-specific ligand, ultra-pure LPS, in mouse RAW264.7, human U-937 and P31/FUJ cells, whereas this agent showed little effect on other TLR ligands, Pam_3CSK_4 (TLR1/2), peptidoglycan (TLR2/6), double strand RNA (TLR3), R-848 (TLR7) and CpG oligonucleotide (TLR9). Furthermore, TAK-242 potently inhibited nuclear factor (NF)-κB activation induced by ultra-pure LPS in HEK293 cells transiently expressing TLR4 and co-receptors, myeloid differentiation protein-2 (MD2) and CD14, whereas this agent showed little effect on other TLRs, TLR1/2, TLR2/6, TLR3, TLR5, TLR7 and TLR9. TAK-242 also inhibited ligand-independent NF-κB activation resulting from over-expression of TLR4. Although chimera receptors, which are consist of the extracellular domain of CD4 and the intracellular domain of human or mouse TLR4, showed constitutive NF-κB activation, TAK-242 potently inhibited the signaling from CD4-TLR4 chimera receptors. In contrast, the NF-κB activation mediated by TLR4 adaptors, myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP), Toll/IL-1R homology (TIR)-domain-containing adaptor protein-inducing interferon-β (TRIF) or TRIF-related adaptor molecule (TRAM) was not affected by TAK-242. TAK-242 is therefore a selective inhibitor of signaling from the intracellular domain of TLR4 and represents a novel therapeutic approach to the treatment of TLR4-mediated diseases.

Keywords: TAK-242; Macrophage; LPS; TLR4; CD4-TLR4, NF-κB; HEK293

1. Introduction

Discovery in the late 1990s of Toll-like receptors (TLRs) as primary sensors of microbial infection led to significant advances in understanding the mechanism of innate immunity. Currently, twelve members of TLRs are identified in mammalian cells (Akira et al., 2006). TLR2 dimerized with TLR1 or TLR6 recognizes peptidoglycan and lipopeptides of gram-positive bacteria (Takeuchi et al., 2002). TLR4 detects Gram-negative bacteria through recognition of the lipid A moiety of lipopolysaccharide (LPS) (Poltorak et al., 1998; Hoshino et al., 1999). LPS recognition is mediated by four molecules, LPS binding protein (LBP), CD14, myeloid differentiation protein-2 (MD2), and TLR4 (Guha and Mackman, 2001). LBP is thought to be a shuttle protein catalyzing LPS transfer from the outer membrane of Gram-negative bacteria to CD14 (Tobias et al., 1995). CD14 has a role in loading LPS to TLR4/MD2 complex. MD-2, a glycoprotein that is essential for the innate response to LPS, binds to both LPS and the extracellular domain of TLR4 (Shimazu et al., 1999). TLR3, 4, 5, 7 and 9 recognize viral double-stranded RNA (Alexopoulou et al., 2001), LPS of gram-negative bacteria (Poltorak et al., 1998; Hoshino et al., 1999), bacterial flagellin (Hayashi et al., 2001), viral single-stranded RNA (Heil et al., 2004; Diebold et al., 2004) and viral and bacterial CpG DNA (Hemmi et al., 2001), respectively. The recognition of microbial pathogens and their components by TLRs triggers the activation of intracellular signaling and results in production of inflammatory mediators such as nitric oxide (NO), prostaglandins,

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doi:10.1016/j.ejphar.2008.01.026

cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, type 1 interferon (IFN) and chemokines.

TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain. After ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of the TIR-domain-containing adaptor molecules to the TIR domain of the TLR (Akira et al., 2006). There are four adaptor molecules, myeloid differentiation factor 88 (MyD88) (Medzhitov et al., 1998), TIR-associated protein (TIRAP) (Yamamoto et al., 2002a; Fitzgerald et al., 2001), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF)/TIR-domain-containing molecule 1 (TICAM1) (Hoebe et al., 2003; Yamamoto et al., 2003; Oshiumi et al., 2003; Sugiyama et al., 2003) and TRIF-related adaptor molecule (TRAM) (Yamamoto et al., 2003). MyD88 is critical for the signaling from TLRs except for TLR3. Upon stimulation, MyD88 associates with the cytoplasmic domain of TLRs and then recruits IL-1R-associated kinase 1 (IRAK-1) and IRAK-4 (Medzhitov et al., 1998; Suzuki et al., 2002; Thomas et al., 1999). They mediate activation of a transcriptional factor, nuclear factor (NF)-κB, resulting in induction of inflammatory mediators. TIRAP mediates MyD88-dependent signaling (Yamamoto et al., 2002a; Fitzgerald et al., 2002), and TRIF is required for MyD88-independent signaling and involved in both TLR3 and TLR4 signaling (Yamamoto et al., 2003; Oshiumi et al., 2003). Upon stimulation, TRIF stimulation triggers the activation of NF-κB and IFN regulatory factor 3 (IRF3), and induces the expression of inflammatory cytokine genes and type 1 IFN gene (Yamamoto et al., 2002b). TRAM, which bridges TLR4 and TRIF, is a specific adaptor for TLR4 and transmits its signaling to TRIF (Yamamoto et al., 2003). Thus, TLRs stimulated with microbial components activate intracellular signaling via the interaction with adaptors and induce production of inflammatory mediators.

The initiation of innate immune response through TLR4 triggers an inflammatory cascade that is the principal cause of harmful conditions such as sepsis. Consequently, TLR4 is a promising therapeutic target for the treatment of sepsis (Opal and Huber, 2002; Lynn et al., 2004). A novel small-molecule antisepsis agent, TAK-242, was originally discovered as an inhibitor of production of inflammatory mediators such as IL-6 and TNF-α from LPS-stimulated macrophage cells (Yamada et al., 2005) and a pivotal clinical trial of TAK-242 is ongoing in severe sepsis.

To clarify the mechanism of action, the effects of TAK-242 on the signaling mediated by TLRs were investigated in this study. Finally we found that TAK-242 selectively suppresses both ligand-dependent and-independent signaling via the intracellular domain of TLR4.

2. Materials and methods

2.1. Materials

TAK-242 (Ethyl (*6R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate)) was synthesized at Takeda Pharmaceutical Company Limited (Osaka, Japan). Ultra-pure LPS (from *Escherichia coli* serotype O111:B4) was purchased from Invivogen (SanDiego, CA). Peptidoglycan (from *Staphylococcus aureus*) was from Fluka (Buchs, Switzerland). Phosphorothioate-stabilized CpG oligonucleotides (ODN) (TCC-ATG-ACG-TTC-CTG-ATG-CT as a ligand for mouse TLR9, TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT as a ligand for human TLR9) were synthesized at Hokkaido System Science (Sapporo, Japan). Flagellin was from Invivogen. Pam_3CSK_4 was from Bachem AG (Bubendorf, Switzerland). Polyinosinic-polycytidylic acid (poly(I:C)) was from Sigma. R-848 was from GLSynthesis Inc. (Worcester, MA). Recombinant mouse IFN-γ was from Genzyme (Minneapollis, MN). Phorbol myristate acetate (PMA) was from Wako (Osaka, Japan).

2.2. Cells

Mouse RAW264.7 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Human U-937 cells were purchased from Dainippon Sumitomo Pharmaceutical (Osaka, Japan). Human P31/FUJ cells were purchased from Japanese Collection of Research Bioresources (Osaka, Japan). These cells were maintained in RPMI-1640 medium supplemented with 10% inactivated fetal calf serum (FCS) and 50 μg/ml gentamycin (Invitrogen, USA). Human embryonic kidney HEK293 cells were purchased from Dainippon Sumitomo Pharmaceutical (Osaka, Japan) and maintained in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% inactivated FCS and 50 μg/ml gentamycin.

2.3. Plasmids

6cDNAs encoding human TLR1, human TLR2, human TLR3, human TLR4, mouse TLR4, human TLR6, human TLR7, human TLR9, human CD14, human MD2, mouse MD2, human MyD88, human TIRAP, human TRIF, and human TRAM were isolated by polymerase chain reaction (PCR) and subcloned into a mammalian expression vector, pcDNA3.1 (Invitrogen, USA). Human TLR5 expression vector, pUNO-human TLR5 was purchased from Invivogen. The transmembrane and the intracellular domain of mouse TLR4 (amino acid residue 623 to 835) or human TLR4 (amino acid residue 632 to 839) were fused to the extracellular domain of mouse CD4 (amino acid residue 1 to 384). The chimera receptors were ligated into a mammalian expression vector pMSRαneo, which contained SRα promoter. All constructions identified by PCR were verified by sequencing.

2.4. Measurement of nitrite

RAW264.7 cells were seeded in 96-well plates at 1×10^5 cells per well. After 24 h culture, cells were incubated with TAK-242 for 1 h, subsequently stimulated with each ligands for TLRs, 1 or 10 ng/ml LPS for TLR4, 10 or 100 ng/ml Pam_3CSK_4 for TLR2/TLR1, 1 or 10 μg/ml Peptidoglycan for TLR2/TLR6, 1 or 10 μg/ml poly(I:C) for TLR3, 1 or 10 μM R-848 for TLR7, or 0.1 or 1 μM CpG ODN for TLR9 in the presence of 0.1 ng/ml

mouse IFN-γ for 24 h. The concentrations of nitrite, a stable metabolite of nitric oxide (NO), in culture medium were determined with a fluorescent reagent, 2,3-diaminonaphthalene (Dojindo, Japan). The fluorescence (Excitation 355 nm, Emission 460 nm) was detected with Arvo 1420 multilabel counter (Perkinelmer, USA). The concentration (IC_{50} value) of the test compound necessary for 50% inhibition of NO production was calculated using the least-squares linear regression.

2.5. Measurement of TNF-α

RAW264.7 cells were seeded in 96-well plates at 1×10^5 cells per well. After culture for 24 h, cells were incubated with TAK-242 for 1 h, subsequently stimulated with 10 ng/ml ultrapure LPS for 24 h. U-937 or P31/FUJ cells were seeded in 96-well plates at 4×10^4 cells per well. After culture for 24 h, cells were added 100 nM PMA (Wako, Japan) for differentiation into macrophages. After culture for 48 h, cells were incubated with TAK-242 for 1 h, subsequently stimulated with 10 ng/ml ultrapure LPS for 24 h. The concentrations of TNF-α in culture medium were determined with human or mouse TNF-α ELISA kits. The concentrations (IC_{50} values) of the test compound necessary for 50% inhibition of TNF-α production were calculated using the least-squares linear regression.

2.6. Reporter gene assay for ligand-dependent signaling by TLRs

HEK293 cells were seeded in 96-well opaque plates at 2×10^4 cells per well. After culture for 24 h, cells were transiently transfected with 30 ng TLR expression vectors or empty vector, 10 ng pNF-κB-luc (Stratagene, USA) and 10 ng an internal control plasmid phRL-TK (Promega) using FuGENE 6 reagent (Roche, USA). For expression of TLR4/MD2/CD14 complex, cells were transiently transfected with 5 ng TLR4 expression vector or empty vector, 10 ng pNF-κB-luc, 10 ng an internal control plasmid phRL-TK, 12.5 ng MD-2 and 12.5 ng CD14 expression vectors using FuGENE 6 reagent. For expression of TLR1/TLR2 and TLR2/TLR6, cells were transiently transfected with each 15 ng TLR expression vectors, 10 ng pNF-κB-luc, 10 ng phRL-TK using FuGENE 6 reagent. After transfection, cells were cultured for 24 h. Then, cells were incubated in serum-free medium and various concentrations of TAK-242 were added to the wells. After incubation for 30 min, cells were stimulated by each TLR ligands, 1 or 10 ng/ml ultrapure LPS for TLR4, 10 or 100 ng/ml Pam_3CSK_4 for TLR2/TLR1, 100 or 1000 ng/ml Peptidoglycan for TLR2/TLR6, 1 or 10 μg/ml poly(I:C) for TLR3, 10 or 100 ng/ml flagellin for TLR5, 1 or 10 μM R-848 for TLR7, or 0.1 or 1 μM CpG ODN for TLR9 and then incubated for 4 h. The luciferase activities were measured using Dual-Glo luciferase assay system (Promega). Transfection efficiencies were normalized to luciferase activity derived from *Renilla reniformis*. The luminescence was detected with Arvo 1420 multilabel counter (Perkinelmer, USA). The concentrations (IC_{50} values) of the test compound necessary for 50% inhibition of NF-κB activity were calculated using the least-squares linear regression.

2.6.1. Reporter gene assay for ligand-independent signaling by TLR4, CD4-TLR or adaptors

HEK293 cells were seeded in 96-well opaque plates at 2×10^4 cells per well. After culture for 24 h, cells were transiently transfected with 30 ng TLR4, CD4-TLR4 or adaptors (MyD88, TRIF, TRAM and TIRAP) expression vector or empty vector, 10 ng pNF-κB-luc and 10 ng an internal control plasmid phRL-TK per well using FuGENE 6 reagent. After transfection, various concentrations of TAK-242 were added to the wells and further incubated for 24 h. The luciferase activities were measured using Dual-Glo luciferase assay system. Transfection efficiencies were normalized to luciferase activity derived from *R. reniformis*. The luminescence was detected with Arvo 1420 multilabel counter (Perkinelmer, USA). The concentrations (IC_{50} values) of the test compound necessary for 50% inhibition of NF-κB activity were calculated using the least-squares linear regression.

2.7. Western blot analysis

Transfected cells were harvested in cell lysis buffer (Cell signaling technology, Inc, USA). After incubation at 4 °C for 15 min, the lysate was mixed with an equal volume of a sample buffer (Daiichi Pure Chemicals Co., Ltd., Japan) and heated at 60 °C for 5 min. Proteins were electrophoresed on SDS-polyacrylamide gels with Tris-glycine running buffer and electrically transferred onto Clear Blot Membrane P (Atto, Japan), which was followed by incubating with 5% (w/v) bovine serum albumin in TBS (tris buffered saline)/Tween20. After incubation at 4 °C overnight, the membrane was washed and then incubated at room temperature with anti-mouse CD4 (Santa Cruz biotechnology, Inc. USA). After incubation for 1 h, the membrane was incubated at room temperature with horseradish peroxidase-conjugated anti-rat IgG (1:2000) for 1 h and then visualized by using the ECL Plus western blotting detection reagents (GE Healthcare, USA). Horseradish peroxidase-conjugated anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz biotechnology, Inc.) was used as an internal control.

2.8. Statistical analysis

Data are expressed as the mean ± S.E.M. Differences between means were analyzed using t-test. IC_{50} values for TAK-242 were determined as the concentration showing 50% of control using the least-squares linear regression. All calculations were carried out with the SAS system.

3. Results

3.1. Selectivity of TAK-242 against TLR ligands in mouse RAW264.7cells

To confirm whether the prevention of LPS-induced inflammation by TAK-242 is caused by inhibition of TLR4 signaling, we examined effects of TAK-242 on signaling mediated by ultra-pure LPS as a TLR4-specific ligand in this study. First, the selectivity of TAK-242 against TLR ligands was determined by

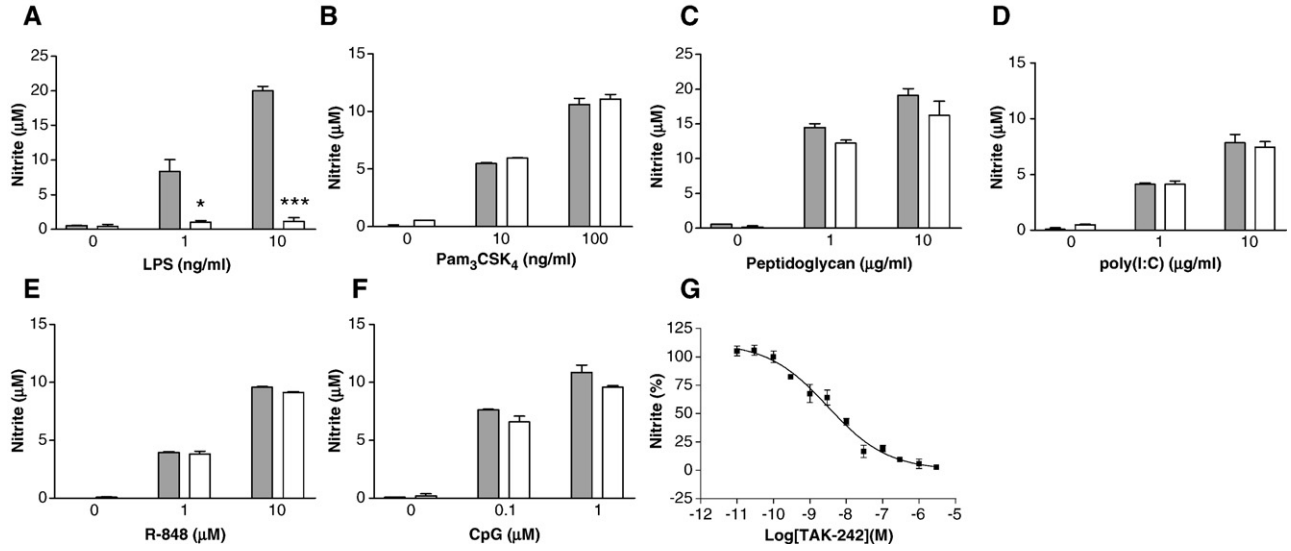

Fig. 1. Effect of TAK-242 on TLR ligands-induced nitric oxide production from mouse RAW264.7 cells. Mouse RAW264.7 cells were stimulated by various TLR ligands, LPS (TLR4 ligand) (A), Pam_3CSK_4 (TLR2/1 ligand) (B), peptidoglycan (TLR2/6 ligand) (C), poly(I:C) (TLR3 ligand) (D), R-848 (TLR7 ligand) (E), or CpG ODN (TLR9 ligand) (F) in the absence or presence of 1 μM TAK-242 (gray bars: vehicle, white bars: 1 μM TAK-242) with 0.1 ng/ml mouse IFN-γ for 24 h. After incubation, the concentrations of released NO in the medium were measured with 2,3-diaminonaphthalene. (G) Dose-dependent inhibition of NO production by TAK-242 was measured with RAW264.7 cells stimulated with 10 ng/ml ultra-pure LPS. Results are the means±S.E.M ($n=3$). *$P<0.05$ and ***$P<0.001$ vs. vehicle.

measuring NO production from mouse RAW264.7 cells. NO production from RAW264.7 cells was induced by TLR ligands, ultra-pure LPS (TLR4-specific ligand), Pam_3CSK_4 (TLR2/1 ligand), peptidoglycan (TLR2/6 ligand), poly(I:C) (TLR3 ligand), R-848 (TLR7 ligand), CpG DNA (TLR9 ligand) with 0.1 ng/ml IFN-γ. TAK-242 selectively inhibited ultra-pure LPS-induced NO production (Fig. 1A). In contrast, TAK-242 showed little effect on NO production induced by TLR2/1, TLR2/6, TLR3, TLR7 or TLR9 ligands at 1 μM (Fig. 1B-F). As shown in Fig. 1G, TAK-242 potently inhibited NO production from RAW264.7 cells stimulated with 10 ng/ml ultra-pure LPS in a dose-dependent manner with an IC_{50} value of 5.5 nM.

3.2. Effect of TAK-242 on TNF-α production from human and mouse macrophages stimulated with a TLR4-specific ligand

To examine effects of TAK-242 on TNF-α production from both human and mouse macrophages stimulated with ultra-pure LPS, the inhibitory activity for TAK-242 was determined with human U-937, human P31/FUJ or mouse RAW264.7 cells. The concentrations of TNF-α released from 10 ng/ml ultra-pure LPS-stimulated U-937, P31/FUJ and RAW264.7 cells were 7.0 ng/ml, 3.5 ng/ml and 3.0 ng/ml, respectively (data not shown). In human U-937 and P31/FUJ cells, TAK-242 concentration-dependently inhibited TNF-α production induced by ultra-pure-LPS with IC_{50} values of 52 nM and 37 nM, respectively (Fig. 2). In mouse RAW264.7 cells, an IC_{50} value of TNF-α production for TAK-242 was 4.8 nM. TAK-242 showed a potent inhibition of inflammatory cytokine production from both mouse and human macrophages stimulated with TLR4-specific ligand, though the inhibitory activity in the mouse macrophage cell line was 7.7 to 11 fold greater than that of the human cell lines.

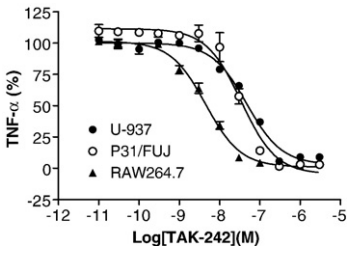

Fig. 2. Effect of TAK242 on LPS-induced TNF-α production from human U-937, P31/FUJ cells and mouse RAW264.7 cells. Monocytic cell lines, human U-937 (closed circle), human P31/FUJ cells (opened circle), or mouse RAW264.7 cells (closed triangle) were incubated with TAK-242 for 1 h, subsequently stimulated with 10 ng/ml ultra-pure LPS for 24 h. The concentrations of TNF-α in the culture medium were determined by ELISA. Results are the means±S.E.M ($n=3$).

3.3. Selectivity of TAK-242 against recombinant TLRs

To confirm our results obtained in macrophages, we constructed an NF-κB reporter gene assay system with HEK293 cells transiently expressing recombinant TLRs, TLR2/l, TLR2/6, TLR3, TLR4, TLR5, TLR7 or TLR9. The expressed TLRs activated NF-κB by adding their ligands (Fig. 3A-H). To detect the activation of TLR4 induced by LPS, its co-receptors, CD14 and MD2, were co-expressed with TLR4. TAK-242 inhibited NF-κB activation induced by ultra-pure LPS in HEK293 cells expressing human TLR4, human MD2 and human CD14 (Fig. 3A) or mouse TLR4, mouse MD2 and human CD14 (Fig. 3B) at 1 μM. In the absence of co-expression of CD14 and MD2, TLR4 was not activated by adding LPS (data not shown). In contrast, TAK-242 did not show inhibition of other TLRs such as TLR2/l, TLR2/6, TLR3, TLR5, TLR7 or TLR9 at 1 μM (Fig. 3C–H). The IC_{50} values of LPS-induced NF-κB

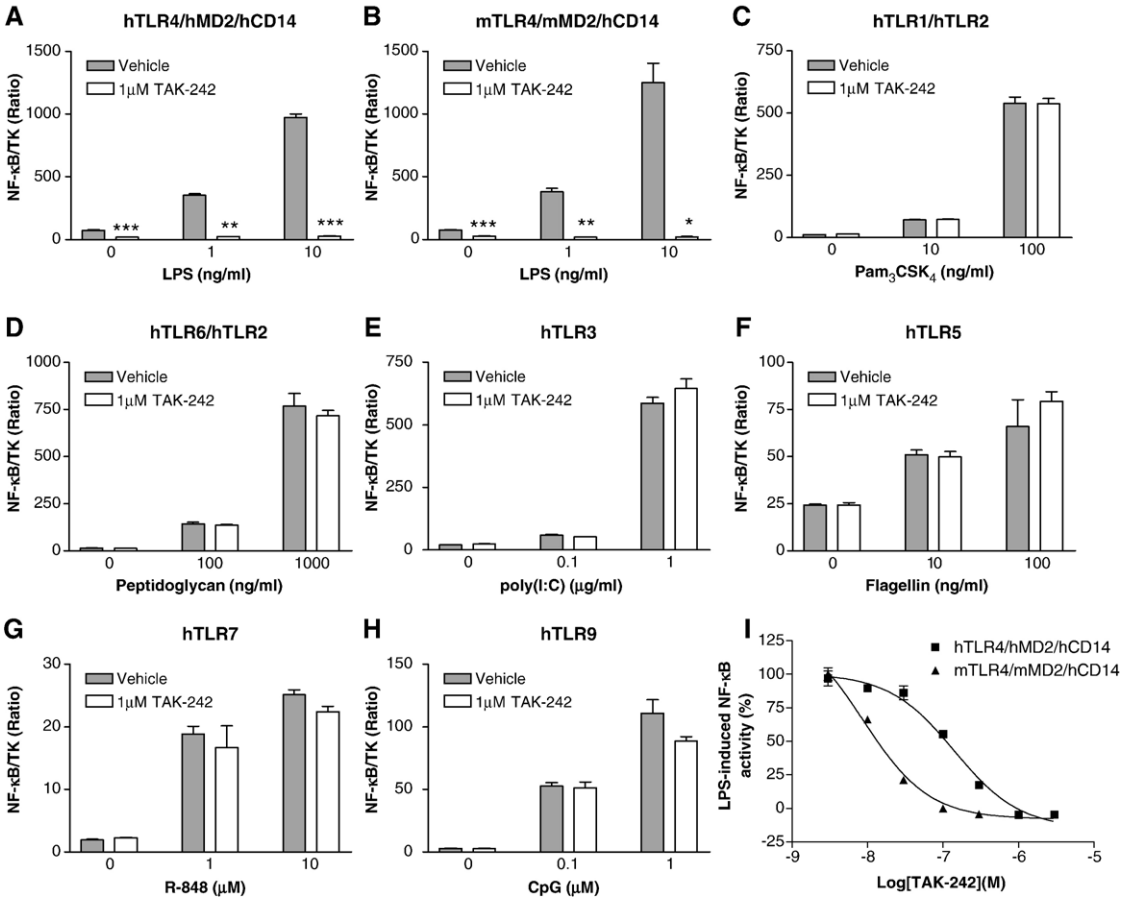

Fig. 3. Effect of TAK-242 on NF-κB activation mediated by recombinant TLRs stimulated with their ligands. HEK293 cells were transiently co-transfected with an NF-κB reporter plasmid, an internal control reporter plasmid phRL-TK and the expression plasmids for TLRs, and then cultured for 24 h. Cells were incubated with DMSO or 1 μM TAK-242 for 30 min, followed by adding their ligands, LPS (TLR4 ligand) (A, B), Pam_3CSK_4 (TLR2/1 ligand) (C), peptidoglycan (TLR2/6 ligand) (D), poly(I:C) (TLR3 ligand) (E), flagellin (TLR5 ligand) (F), R-848 (TLR7 and 8 ligand) (G), or CpG ODN (TLR9 ligand) (H) in the absence or presence of 1 μM TAK-242 (gray bars: vehicle, white bars: 1 μM TAK-242). After 4 h culture, cells were lysed and the NF-κB reporter activities were measured using Dual-Glo luciferase assay system. Promoter activities were normalized to Renilla luciferase activities. (I) Dose-dependent inhibition of NF-κB activation stimulated with 10 ng/ml LPS by TAK-242 was examined in HEK293 cells transiently expressing hTLR4/hMD2/hCD14 (closed square) or mTLR4/mMD2/hCD14 (closed triangle). Results are the means ± S.E.M ($n=3$). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. vehicle.

activation for TAK-242 in HEK293 cells expressing human TLR4, human MD2 and human CD14 or mouse TLR4, mouse MD2 and human CD14 were 110 nM and 15 nM, respectively (Fig. 3I). This result corresponds that TAK-242 showed more potent inhibition of mediators production from LPS-stimulated mouse cells than human cells. TAK-242 showed a potent inhibition of signaling from both mouse and human TLR4 stimulated with its ligand.

3.4. Effect of TAK-242 on ligand-independent signaling of TLR4

TLR4 has been shown to activate ligand-independent signaling (Lee et al., 2004). As shown in Fig. 4A, both human and mouse TLR4 showed a constitutive activation of NF-κB in the absence of expression of MD2 and CD14. The effect of TAK-242 on ligand-independent signaling of TLR4 was examined with this assay system. TAK-242 potently inhibited the ligand-independent activation of NF-κB mediated by over-expression of human or mouse TLR4 at 1 μM. This result indicated that TAK-242 does not affect the function of MD2 or CD14. The IC_{50} values of ligand-independent NF-κB activation by human TLR4, or mouse TLR4 for TAK-242 were 310 nM and 35 nM, respectively (Fig. 3B). Thus, TAK-242 potently suppressed ligand-independent signaling from both mouse and human TLR4.

3.5. Effect of TAK-242 on NF-κB activation mediated by adaptors for TLR4

After ligand binding, the intracellular signaling of TLR4 is mediated by its adaptor proteins such as MyD88, TIRAP, TRIF and TRAM (Akira et al., 2006). Expression of these adaptors in cells has been shown to result in activation of their down stream signaling. To examine effects of TAK-242 on MyD88, TIRAP, TRIF and TRAM, the signaling activated by transiently expression of adaptors for TLR4 in HEK293 cells was measured with NF-κB reporter gene assay. As a result, transfection of expression plasmids for MyD88, TIRAP, TRIF or TRAM to HEK293 cells led to marked activation of NF-κB

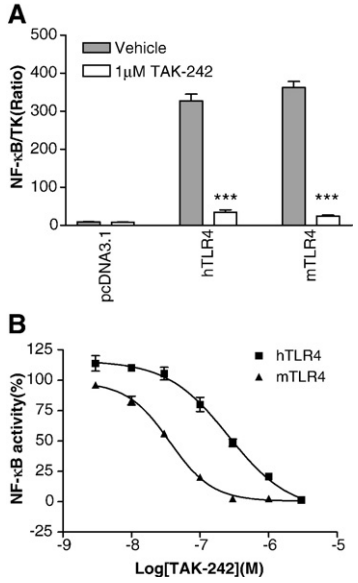

Fig. 4. Effect of TAK-242 on ligand-independent signaling of TLR4. (A) HEK293 cells were transiently co-transfected with an NF-κB reporter plasmid, an internal control reporter plasmid phRL-TK and the expression plasmid for human TLR4 or mouse TLR4, or the empty plasmid pcDNA3.1 and further cultured for 24 h. (B) After transfection, cells were cultured in the presence of various concentrations of TAK-242. After 24 h culture, cells were lysed and the NF-κB reporter activities induced by human TLR4 (closed square) or mouse TLR4 (closed triangle) were measured using Dual-Glo luciferase assay system. Promoter activities were normalized to Renilla luciferase activities. Results are the means±S.E.M ($n=3$). ***$P<0.001$ vs. vehicle.

compared with the empty vector. However, TAK-242 did not affect the signaling from those adaptors at 1 μM (Fig. 5).

3.6. Effect of TAK-242 on NF-κB activation mediated by CD4-TLR4 chimera receptor

To estimate the interacting region of TLR4 with TAK-242, the effects of TAK-242 on CD4-TLR4 chimera receptor, which is consist of the extracellular domain of CD4 and the intracellular domain of TLR4, were examined with NF-κB reporter gene assay. The chimera receptors of CD4-human TLR4 or CD4-mouse TLR4 potently activated NF-κB in transiently expressed HEK293 cells (Fig. 6A). The NF-κB activation mediated by CD4-human TLR4 or CD4-mouse TLR4 was concentration-dependently inhibited by TAK-242. The IC_{50} values of CD4-human TLR4 and CD4-mouse TLR4 for TAK-242 were 240 nM and 62 nM, respectively (Fig. 6B). To examine whether TAK-242 affects the expression of chimera receptors in transfected HEK293 cells, western blot analysis was performed with antibody that recognizes the extracellular domain of CD4. As shown in Fig. 6C, this compound did not affect the expression levels of CD4-human TLR4 and CD4-mouse TLR4 at 1 μM. Thus, TAK-242 showed a potent inhibition of signaling mediated by the intracellular domain of TLR4.

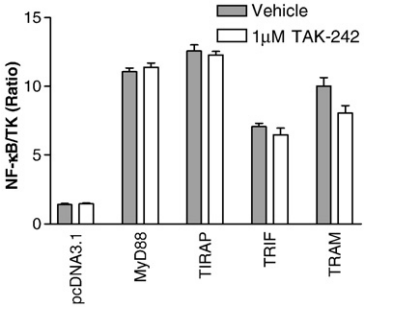

Fig. 5. Effect of TAK-242 on NF-κB activation mediated by adaptors for TLR4. HEK293 cells were transiently co-transfected with an NF-κB reporter plasmid, an internal control reporter plasmid phRL-TK and the expression plasmids for adaptors, human MyD88, human TIRAP, human TRIF or human TRAM, or empty vector. After transfection, cells were cultured in the absence or presence of 1 μM TAK-242 (gray bars: vehicle, white bars: 1 μM TAK-242) for 24 h. After culture, the NF-κB reporter activities were measured using Dual-Glo luciferase assay system. Promoter activities were normalized to Renilla luciferase activities. Results are the means±S.E.M ($n=3$).

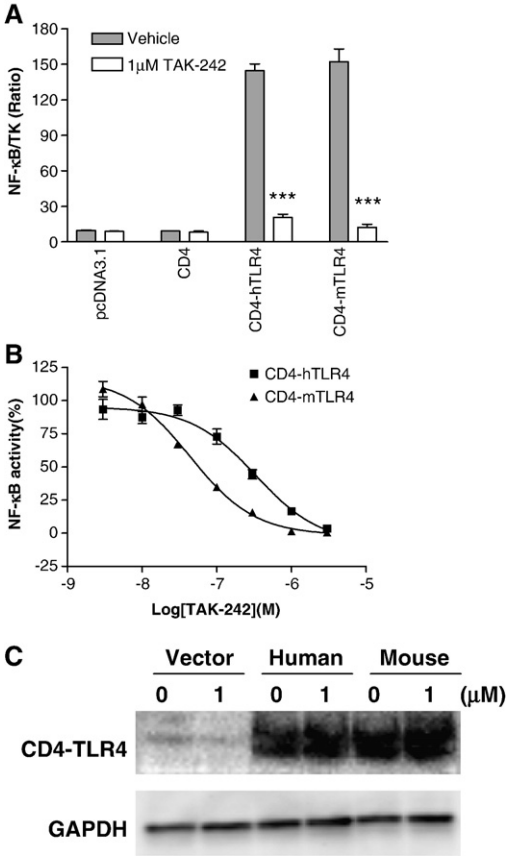

Fig. 6. Effect of TAK-242 on ligand-independent signaling by CD4-TLR4 chimera receptor. (A) HEK293 cells were transiently co-transfected with an NF-κB reporter plasmid, an internal control reporter plasmid phRL-TK and the expression plasmid for CD4-humanTLR4, CD4-mouseTLR4, or CD4 and further cultured for 24 h. (B) After transfection, cells were cultured in the presence of various concentrations of TAK-242. After 24 h culture, cells were lysed and the NF-κB reporter activities induced by CD4-human TLR4 (closed square) or CD4-mouse TLR4 (closed triangle) were measured using Dual-Glo luciferase assay system. Promoter activities were normalized to Renilla luciferase activities. Results are the means±S.E.M ($n=3$). ***$P<0.0001$ vs. vehicle. (C) Expression of CD4-human TLR4 or CD4-mouse TLR4 in transfected HEK293 cells in the absence or presence of 1 μM TAK-242 was analyzed with western blotting using anti-CD4 antibody. Protein loading was controlled by running GAPDH western blotting.

4. Discussion

The pathogenesis of sepsis involves a progressive and dynamic expansion of the systemic inflammatory response to microbial infection (Glauser, 2000). A major factor contributing to sepsis is the shedding of LPS from the cell wall of Gram-negative bacteria into the circulation. LPS interacts with TLR4 expressed in a variety of cell types and induces hyperproduction of various cytokines. Systemic release of cytokines induces vascular permeability and disseminated intravascular coagulation, which often leads to a state of shock (Annane et al., 2005). In previous reports, we showed TAK-242 suppressed LPS-induced inflammation *in vitro* and *in vivo* (Ii et al., 2006; Sha et al., in press). TAK-242 might be a promising drug for the suppression of excessive inflammatory response at the first line by inhibition of TLR4 signaling, but its mechanism of action has not fully been elucidated. Most LPS preparations on the market are contaminated by other bacterial components, such as lipoproteins, thus activating TLR2 signaling as well as TLR4 signaling. To clarify the effect of TAK-242 on TLR4 signaling, we used ultra-pure LPS, which was extracted by enzymatic hydrolysis and purified with the phenol re-extraction (Hirschfeld et al., 2000), thus selectively activating the TLR4 pathway and TLRs expression systems. Our findings support that TAK-242 is a selective inhibitor of TLR4 signaling, which also resulted in its efficacy against LPS-induced inflammation.

LPS induces the dimerization of TLR4, and then TLR4 confers the ligand-independent activation of the receptor. The receptor dimerization is required to activate downstream signaling pathways (Lee et al., 2004). In this study, we demonstrated that TAK-242 potently suppresses both ligand-dependent and-independent signaling of TLR4 (Figs. 3 and 4). These results were consistent with our previous study that TAK-242 does not inhibit an interaction between LPS and its receptor in macrophages. Furthermore, our results from the NF-κB reporter gene assay indicated that TLR4 shows a more potent constitutive activity than other TLRs. Expression of TLR4 is induced by the treatment with various stimulants such as IFN-γ (Faure et al., 2001), IL-10 (Petit-Bertron et al., 2003), IL-2 (Mita et al., 2002) or oxidized low-density lipoprotein (OxLDL) (Xu et al., 2001). In addition, TLR4 is persistently expressed at high levels in atherosclerosis (Edfeldt et al., 2002) and inflammatory bowel diseases (IBD) (Cario and Podolsky, 2000). Over-expression of TLR4 has indeed been found to activate NF-κB in the absence of TLR4 ligands (Fitzgerald et al., 2001). Thus, individuals who tend to exhibit enhanced or persistent TLR4 expression may be more susceptible to chronic inflammatory conditions. TAK-242 might also show an efficacy against inflammation mediated by excessive expression of TLR4. Furthermore, TLR4 has been shown to respond to various endogenous ligands such as heat-shock proteins (HSP60, HSP70, gp96 and HSP22), extracellular matrix (ECM) degradation products (Biglycan, hyaluronan, fibronectin extradomain A and surfactant protein-A), miscellaneous (high-mobility group box 1 (HMGB-1), OxLDL and β-defensin), (Miyake, 2007) and saturated free fatty acids (Lee et al., 2001). Interaction of TLR4 with endogenous ligands is involved in progression of various human diseases, arthritis, asthma, atherosclerosis and diabetes etc. (Cook et al., 2004; Shi et al., 2006). Indeed, TLR4 deficiency is associated with protection against high fat diet-induced insulin resistance in mice (Shi et al., 2006) and reduction in aortic atherosclerosis in apolipoprotein E deficient mice (Michelsen et al., 2004). Recent studies indicate that TLR4 polymorphisms might affect atherogenesis (Cook et al., 2004). A report from the Stockholm Heart Epidemiology Program suggests that hypo-responsive TLR4 polymorphisms affect the susceptibility to myocardial infarction in men and that TLR4-mediated innate immunity plays a role in the pathogenesis of myocardial infarction (Edfeldt et al., 2004). TAK-242 may also prevent the progression of TLR4-mediated various diseases since TAK-242 inhibits the intracellular signaling of TLR4.

After ligand binding, TLR4 recruits the TIR-domain-containing adaptor molecules, MyD88, TIRAP, TRIF and TRAM, to the TIR domain of the TLR4 (Akira et al., 2006). Although the responses to IL-1 and various TLR agonists are completely impaired in MyD88-deficient mice, for LPS-TLR4 some signals are intact, such as late-phase NF-κB activation and stimulation of the interferon pathway through IRF3 activation (Kawai et al., 2001). TIRAP is a TLR4 adaptor for MyD88-independent signaling (Yamamoto et al., 2002a; Fitzgerald et al., 2002). Although TRIF activates NF-κB, it also induces the expression of the gene encoding interferon-β (Yamamoto et al., 2002b; Oshiumi et al., 2003; Sugiyama et al., 2003). TRAM is a specific adaptor for TLR4 and triggers MyD88-independent signaling (Yamamoto et al., 2003). Although TAK-242 inhibited production of various mediators from LPS-stimulated macrophages and TLR4-mediated NF-κB activation, this agent did not affect signaling mediated by over-expression of adaptors for TLR4 such as MyD88, TIRAP, TRIF and TRAM (Fig. 5). This result suggests that TAK-242 targets the upstream molecules of the adaptors including TLR4 itself except for these four TLR adaptors.

TLR signaling is conferred on TIR domain. The signaling mediated by CD4-TLR4 TIR domain chimera receptor was also potently inhibited by TAK-242 (Fig. 6). These results suggest that TAK-242 inhibits the signaling mediated by the intracellular domain of TLR4, but not the extracellular domain. TAK-242 showed more potent inhibition of mediator production from LPS-stimulated mouse macrophages than human macrophages. This species difference in the inhibitory activity for TAK-242 is consistent with the reporter gene assays using recombinant TLR4 and our previous study with primary cultured cells (Ii et al., 2006). The inhibitory activity for TAK-242 was changed simply by replacement of TIR of TLR4 under the same cellular condition, whereas the induction of NF-κB activity by mouse TLR4 was at same level as that by human TLR4. This result raises the possibility that TAK-242 targets TIR of TLR4. Indeed, a single-point mutation in the TIR domain of mouse TLR4 (Pro712His, the Lps(d) mutation) abolishes the host immune response to LPS (Xu et al., 2000). Thus, it seems possible that a small molecule like TAK-242 can suppress the function of TLR4 by binding to or modifying single amino acid. Although the amino acid sequence of TIR between human and mouse TLR4 is conserved, the interacting affinity of TAK-242 with TLR4 may be affected by such a subtle difference in the amino acid sequences of TIR. A

TLR4 antagonist, E5531, preferentially blocks LPS interaction with TLR4-MD-2, but shows a weak effect on blocking LPS interaction with CD14 (Akashi et al., 2003). Thus, TAK-242, which can also inhibit TLR4-mediated signaling even in the absence of MD2 and CD14, is expected to have more potential to suppress inflammation induced by various TLR4 ligands than TLR4 antagonists. Sepsis is a leading killer in the noncoronary intensive care unit, and it remains worldwide health concerns (Martin et al., 2003; Strehlow et al., 2006). TAK-242 might be a promising novel class of therapeutic agent for the treatment of sepsis.

In conclusion, we demonstrated that TAK-242 selectively suppresses TLR4-signaling mediated by the intracellular domain. Recent findings suggest that TLR4 signaling pathways might be related to human diseases involving innate immunity, adaptive immunity and both innate and adaptive immunity (Cook et al., 2004). Manipulation of TLR4 pathways is considered to have great therapeutic potential. TAK-242 represents a novel therapeutic approach to the treatment of TLR4-mediated diseases. It is unclear whether TAK-242 directly inhibits the dimerization of TLR4 or the interaction of TLR4 with its adaptors. Thus, further analysis would be required to clarify the more precise mechanism of action.

Acknowledgements

We thank T. Itoh, M. Mochizuki, K. Takashima, T. Ichikawa and N. Matsunaga for helpful discussions and C. Kawahara for her technical assistance.

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