



Title	Apoptosis signal-regulating kinase 1-mediated sustained p38 mitogen-activated protein kinase activation regulates mycoplasmal lipoprotein- and staphylococcal peptidoglycan-triggered Toll-like receptor 2 signalling pathways
Author(s)	Into, Takeshi; Shibata, Ken-ichiro
Citation	Cellular Microbiology, 7(9), 1305-1317 https://doi.org/10.1111/j.1462-5822.2005.00558.x
Issue Date	2005-09
Doc URL	https://hdl.handle.net/2115/30270
Rights	The definitive version is available at www.blackwell-synergy.com
Type	journal article
File Information	CM7-9.pdf



CMI-05-0056

Apoptosis signal-regulating kinase 1 -mediated sustained p38 mitogen-activate protein kinase activation regulates mycoplasmal lipoprotein- and staphylococcal peptidoglycan-triggered Toll-like receptor 2 signaling pathways

Takeshi Into,[†] and Ken-ichiro Shibata*

*Laboratory of Oral Molecular Microbiology, Department of Oral Pathobiological Science,
Hokkaido University Graduate School of Dental Medicine, Nishi 7, Kita 13, Kita-ku, Sapporo
060-8586, Japan*

*For correspondence: Ken-ichiro Shibata, Department of Oral Pathobiological Science, Hokkaido University Graduate School of Dental Medicine, Nishi 7, Kita 13, Kita-ku, Sapporo 060-8586, Japan.

E-mail: shibaken@den.hokudai.ac.jp; Tel. +81(11)706-4240. Fax +81(11)706-4901.

[†]Present address: Laboratory of Oral Disease Research, National Institute for Longevity Sciences (NILS), Aichi, Japan

Running title: Roles of ASK1 in TLR2 signaling

Key words: TLR2; ASK1; p38 MAPK; AP-1; NF- κ B; caspase

Summary

Toll-like receptor (TLR) 2 functions as a sensor for detecting various microbial components conserved in bacteria or fungi in innate immunity. TLR2 induces several signaling pathways linking to activation of the transcriptional factors NF- κ B and AP-1 as well as induction of cell death. In human embryonic kidney 293 cells expressed human TLR2, mycoplasmal lipoproteins (MLP) or staphylococcal peptidoglycans (PGN) induced sustained phosphorylation of p38 MAPK, accompanied by generation of reactive oxygen species. This observation encouraged us to examine roles of apoptosis signal-regulating kinase 1 (ASK1) in TLR2 signaling, because ASK1 is an upstream activator of p38 MAPK during exposure to oxidative stress and other stressful stimuli. A kinase-inactive mutant of ASK1 greatly impaired the sustained phosphorylation of p38 MAPK induced by MLP or PGN. This mutant also attenuated MLP- or PGN-induced transcriptional activities of NF- κ B and AP-1 via inhibition of p38 MAPK activation. MLP- or PGN-induced cell death reactions, including DNA fragmentation and caspase-3/7 activation, were also downregulated by the ASK1 mutant via p38 MAPK inhibition. Furthermore, TLR2 signaling had a potential to phosphorylate and dephosphorylate ASK1 at Ser83 residue. Thus, MLP and PGN have capabilities to induce ASK1-dependent signaling pathways which regulate p38 MAPK activation through TLR2, leading to activation of NF- κ B and AP-1 as well as induction of cell death.

Introduction

Toll-like receptors (TLRs) represent a family of type I transmembrane proteins characterized by multiple copies of leucine-rich repeats in the extracellular domain and a cytoplasmic Toll/IL-1R homology (TIR) domain (Medzhitov *et al.*, 1997). At least eleven TLRs have so far been identified in mammalian species, and they are capable of sensing broad classes of pathogens, including viruses, bacteria, fungi and protozoan parasites, by recognizing of specific and distinct pathogen-associated molecular patterns (PAMPs) as cognate ligands (Akira and Takeda, 2004; Janeway and Medzhitov, 1999; Takeda *et al.*, 2003; Zhang *et al.*, 2004a). Among all members of the TLR protein family, TLR2 detects the broadest spectrum of agonistic PAMPs from viruses to protozoans, such as several bacterial lipoproteins/lipopeptides, peptidoglycans (PGN), lipoarabinomannans, porins, glycoproteins and fimbriae, fungal zymosan, viral core proteins and hemagglutinins (HA) and protozoan glycosylphosphatidylinositol anchors (Kirschning and Schmann, 2002; Takeda *et al.*, 2003).

It has been shown that TLR2 first initiates signaling by recruiting the TIR domain-containing adaptors TIRAP (also known as Mal) and MyD88 after recognition of cognate PAMPs (Akira and Takeda, 2004; Horng *et al.*, 2002; Mansell *et al.*, 2004; O'Neill *et al.*, 2003; Yamamoto *et al.*, 2002). The receptor complex is then joined by the members of IL-1R-associated kinases (IRAKs) IRAK1 and IRAK4 via their death domains (DDs) (Akira and Takeda, 2004; Janssens and Beyaert, 2003; Suzuki *et al.*, 2002). After phosphorylation, IRAK1 and IRAK4 dissociate from TLR-associated receptor complex and then interact with TNFR-associated factor (TRAF) 6 (Akira and Takeda, 2004). TRAF6 possesses a unique molecule-binding specificity, especially for TAK1 and TAB1/2, that results in triggering

activation of the cascades of transcriptional factors NF- κ B and AP-1 families (Akira and Takeda, 2004; Bradley and Pober, 2001; Ninomiya-tsuji *et al.*, 1999; Shuto *et al.*, 2001; Takaesu *et al.*, 2000). It is now thought that TRAF6 is a key mediator in the MyD88-dependent TLR/IL-1R signaling pathway leading to activation of a wide variety of innate immune responses (Gohda *et al.*, 2004). MyD88 is also known to recruit a well-known death receptor's adaptor, Fas-associated death domain (FADD), downstream of TLR2 via DD-DD interaction (Aliprantis *et al.*, 2000). After interaction with MyD88, FADD is thought to activate a death initiator cysteine protease, caspase-8, to initiate subsequent proapoptotic reactions. Indeed, we have recently shown that mycoplasmal lipoproteins (MLP) induce apoptotic cell death via MyD88, FADD and caspase-8 downstream of TLR2 (Into *et al.*, 2002b and 2004). Thus, MyD88 can also initiate the so-called death signaling pathway. However, the implication of MyD88 dependence of these partially ambivalent signaling pathways is still controversial. Additionally, several MyD88-independent pathways, including Rac-1, RhoA, protein kinase C, PI3K and Akt/protein kinase B, have also been shown to participate in TLR2-mediated signaling (Arbibe *et al.*, 2000; Strassheim *et al.*, 2004; Teusch *et al.*, 2004).

One of the critical functions of TRAF6 protein is to mediate the activation of AP-1, the activity of which is stimulated by mitogen-activated protein kinases (MAPKs) through either direct phosphorylation or transcription of the AP-1 components c-fos and c-jun (Bradley and Pober, 2001; Dong *et al.*, 2002; Tanoue and Nishida, 2003). MAPKs, which include Ser/Thr kinases such as p38 MAPKs, JNKs and ERKs, are located at the downstream end of a three-tiered system that also contains MAPK kinases (MAPKKs) and MAPK kinase kinases (MAPKKKs). Among MAPK members, p38 MAPKs and JNKs are known as stress-activated protein kinases that are activated by a wide variety of stressors. JNKs are activated by at least

two MAPKKs, SEK1/MKK4 and MKK7, whereas p38 MAPKs are activated by MKK3 and MKK6 (Dong *et al.*, 2002; Tanoue and Nishida, 2003). The activation of these two MAPK cascades is involved in the promotion of both cell survival and apoptotic cell death (Dong *et al.*, 2002; Takeda *et al.*, 2002; Tanoue and Nishida, 2003). Although we recently raised the possibility that TLR2-mediated p38 MAPK activation participated in MLP-induced apoptotic cell death (Into *et al.*, 2004), the regulatory mechanisms and detail functions of MAPKs in TLR2-mediated cell death reactions have not been clarified yet.

In this study, we found that one of the MAPKKK members, apoptosis signal-regulating kinase 1 (ASK1; also known as MEKK5), the activation of which is associated with the generation of intracellular reactive oxygen species (ROS), is a key mediator in regulation of the MLP- or PGN-induced p38 MAPK activation downstream of TLR2. We further show that ASK1 is required for the regulation of MLP- or PGN-induced cell death reactions as well as for the activation of transcriptional activity of AP-1 and NF- κ B downstream of TLR2.

Results

MLP- or PGN-induced phosphorylation of stress-activated MAPKs

MLP and PGN are known as crude immunostimulatory compounds and function as potent agonistic ligands for TLR2. To analyze TLR2-specific signaling events induced by these components, we utilized human embryonic kidney (HEK) 293 cell-derived transfectants, because this cell line lacks endogenous TLR2 expression and is nonresponsive to at least MLP and PGN until TLR2 gene transfection (Fujita *et al.*, 2003; Into *et al.*, 2004). MLP or PGN activated the transcriptional activities of both AP-1 and NF- κ B in HEK293 cells stably transfected with a construct of C-terminal V5 epitope-tagged TLR2 cloned into the pEF6 vector (293/TLR2-V5 cells) in a dose-dependent manner (Fig. 1A and B). Expectedly, HEK293 cells stably transfected with an empty pEF6 vector (293/pEF6 cells) did not respond to both MLP and PGN at the all concentrations used (Fig. 1A and B). 293/TLR2-V5 cells also responded to zymosans as another TLR2 ligand, but the activity was extremely weak when compared with those of MLP and PGN.

Using 293/TLR2-V5 cells, we then examined the phosphorylation state of the stress-activated MAPKs p38 MAPK and JNK by Western blot analyses. MLP induced the phosphorylation of both p38 MAPK and JNK at all concentrations used (Fig. 1C). The phosphorylation of p38 MAPK was detected 15 min after stimulation, reached a maximum at 30 min, and was then gradually reduced (Fig. 1C). The phosphorylation of JNK was detected 60, 30 and 15 min after stimulation with 0.1, 1 and 10 $\mu\text{g ml}^{-1}$ of MLP, respectively, reached a maximum at 30 to 60 min, and was then gradually reduced (Fig. 1C). MLP induced robust phosphorylation of these MAPKs at concentrations of 1 and 10 $\mu\text{g ml}^{-1}$, and the phosphorylation

state was sustained for at least 180 min after stimulation (Fig. 1C). Similar results for PGN-induced phosphorylation of p38 MAPK and JNK were also obtained in the cells (data not shown). Furthermore, we confirmed that $10\ \mu\text{g ml}^{-1}$ of MLP or PGN induced sustained phosphorylation of p38 MAPK and JNK in promonocytic THP-1 cells used as a non-transfected cell line, which constitutively express a functional TLR2 (Into *et al.*, 2002a) (data not shown).

Thus, MLP and PGN activate TLR2-mediated stress-activated MAPKs, and the higher concentration of these compounds could induce sustained phosphorylation of these MAPKs.

Induction of TLR2-mediated oxidative stress by MLP or PGN

It has been reported that various kinds of cellular stressors can induce generation of intracellular ROS. The ROS-associated redox systems are known to affect activation of MAPKs, especially p38 MAPK and JNK (Martindale and Holbrook, 2002; Takeda *et al.*, 2002; Torres and Forman, 2003). Furthermore, it has been reported that stimulation of THP-1 cells with TLR2 ligands induces ROS generation (Aliprantis *et al.*, 1999). Therefore, flow cytometric analysis was carried out to determine whether challenge with MLP or PGN induced ROS generation in 293/TLR2-V5 cells. The stimulation of the cells induced a significant increase in CM-H₂DCFDA fluorescence after 1-h stimulation with MLP or PGN in a dose-dependent manner (Fig. 2), indicating that both MLP and PGN have a capability to induce the generation of intracellular ROS. MLP and PGN did not induce ROS generation in 293/pEF6 cells (data not shown). Thus, challenge with TLR2 ligands induces oxidative stress in TLR2-expressing cells.

Regulation of MLP- or PGN-induced sustained p38 MAPK phosphorylation by ASK1

ASK1 was first discovered by Ichijo *et al.* (Ichijo *et al.*, 1997) as a MAPKKK member that mediated activation of p38 MAPK and JNK and apoptosis in response to oxidative stress and TNF via its catalytic functions. By using ASK1^{-/-} cells, their additional studies have demonstrated that ASK1 was required for sustained activation of p38 MAPK and JNK induced by oxidative stress and TNFR1 (Takeda *et al.*, 2002; Tobiume *et al.*, 2001). Thus, ASK1 has been thought to be an important molecule regulating stress-activated protein kinase pathways and proapoptotic pathways downstream of TNFR1. Importantly, it has been suggested that the TLR2 signaling pathways are analogous to those triggered by TNFR1 (Aliprantis *et al.*, 2000; Into *et al.*, 2004). As described above, this study demonstrated that TLR2 stimulation induced sustained activation of p38 MAPK and JNK and oxidative stress. Therefore, we examined whether ASK1 was involved in MLP- or PGN-induced TLR2 signaling pathways.

The expression of endogenous ASK1 was observed in both parental HEK293 cells and 293/TLR2-V5 cells (Fig. 3A). We constructed a kinase-inactive mutant of ASK1 cloned in the pcDNA3 vector (Lys709Met; ASK1-KM) and stably transfected the construct into 293/TLR2-V5 cells (293/TLR2-V5/ASK1-KM cells; Fig. 3A). 293/TLR2-V5 cells stably transfected with a pcDNA3 empty vector (293/TLR2-V5/pcDNA3 cells) were also established as control cells. Stimulation of 293/TLR2-V5/ASK1-KM cells with MLP (0.1, 1 and 10 $\mu\text{g ml}^{-1}$) resulted in significantly impaired phosphorylation states of both p38 MAPK and JNK when compared with those in 293/TLR2-V5/pcDNA3 cells (Fig. 3B and C; $P < 0.01$). Notably, MLP-induced sustained phosphorylation of p38 MAPK was diminished, but the transient phosphorylation still occurred in 293/TLR2-V5/ASK1-KM cells (Fig. 3B). In contrast to this, MLP-induced sustained JNK phosphorylation was still observed in the cells (Fig. 3C). To

confirm whether the effects of ASK1-KM on phosphorylation of these MAPKs were specific, we then performed experiments using THP-1 cells stably transfected with ASK1-KM (THP-1/ASK1-KM cells) and pcDNA3 empty vector (THP-1/pcDNA3 cells). Stimulation of THP-1/ASK1-KM cells with $10\ \mu\text{g ml}^{-1}$ of MLP resulted in impaired sustained p38 MAPK phosphorylation, which was occurred 1 h after stimulation, when compared with those in THP-1/pcDNA3 cells (Fig. 3D). Distinct differences between pcDNA3 and ASK1-KM were not observed in MLP-induced JNK phosphorylation.

These observations suggest that ASK1 is at least an important molecule to mediate the sustained p38 MAPK phosphorylation downstream of TLR2 via its catalytic functions.

Regulation of MLP- or PGN-induced activation of AP-1 and NF- κ B by ASK1

We next investigated whether ASK1 regulated MLP- or PGN-induced activation of AP-1 and NF- κ B. The activity of AP-1 was significantly decreased in MLP- or PGN-stimulated 293/TLR2-V5/ASK1-KM cells compared with that in 293/TLR2-V5/pcDNA3 cells (Fig. 4A, left panel). It was likely that this decrease was partially due to the decrease in the ASK1-mediated p38 MAPK activation, because the specific inhibitor for p38 MAPK SB203580 slightly decreased AP-1 activity induced by $10\ \mu\text{g ml}^{-1}$ of MLP (Fig. 4A, right panel). MLP-induced JNK activation also participated in the AP-1 activation, because the specific inhibitor for JNK SP600125 partially decreased the activity (Fig. 4A, right panel). Furthermore, we found that the activity of NF- κ B was also significantly decreased in MLP- or PGN-stimulated 293/TLR2-V5/ASK1-KM cells compared with that in 293/TLR2-V5/pcDNA3 cells (Fig. 4B, left panel). This decrease was mainly due to the decrease in ASK1-mediated p38

MAPK activation, but JNK activation, because SB203580, rather than SP600125, showed greater inhibition of NF- κ B activity induced by 10 μ g ml⁻¹ of MLP (Fig. 4B, right panel).

Thus, ASK1 was found to regulate TLR2-mediated activation of p38 MAPK via its catalytic function leading to activation of two major transcriptional factors, AP-1 and NF- κ B.

Regulation of MLP- or PGN-induced cell death by ASK1

TLR2-mediated signaling pathways are linked to the death signal pathway (Aliprantis *et al.*, 2000; Into *et al.*, 2004). We have recently reported that MLP induced several caspase-dependent cell death reactions such as apoptotic morphological changes, externalization of phosphatidylserine, DNA fragmentation and cleavage of poly(ADP-ribose)polymerase through TLR2-, MyD88-, FADD- and p38 MAPK-dependent machineries (Into *et al.*, 2004). In addition, PGN (and MLP, but not zymosan) also induced TLR2-, caspase-8- and caspase-3-dependent cell death in THP-1 cells (Fig. 5A) and other cells (Abrahams *et al.*, 2004). Therefore, experiments were carried out to determine whether ASK1 is involved in MLP- or PGN-induced cell death reactions, especially activation of caspases. Stimulation of 293/TLR2-V5/pcDNA3 cells with MLP or PGN induced DNA fragmentation in a dose-dependent manner (Fig. 5B). Activations of caspase-8, caspase-9 and caspase-3/7 were also clearly observed in the stimulated cells (Fig. 5D-F, left panels). On the other hand, stimulation of 293/TLR2-V5/ASK1-KM cells with MLP or PGN resulted in impaired cell death reactions, mainly in DNA fragmentation and caspase-3/7 activation, and partially in caspase-8 and -9 activations (Fig. 5C-F, left panels). Thus, the catalytic activity of ASK1 is also important for the regulation of TLR2-mediated apoptotic reactions. Notably, pretreatment of 293/TLR2-V5/pcDNA3 cells with SB203580 mainly

attenuated MLP-induced DNA fragmentation and caspase-3/7 activation, whereas pretreatment of the cells with SP600125 mainly attenuated the activations of caspase-8 and caspase-9 (Fig. 5C-F, right panels).

Thus, ASK1 is likely to play important roles in MLP- or PGN-induced cell death reactions mainly by regulation of p38 MAPK-mediated caspase-3 activation downstream of TLR2.

Regulation of phosphorylation of ASK1 by MLP-induced TLR2 signaling

This study demonstrated that the catalytic activity of ASK1 played an important role in the regulation of MLP- or PGN-induced stress and death signaling downstream of TLR2. Recent findings have suggested that the potential of catalytic activity of ASK1 is neutralized by the phosphorylation of several serine residues, including Ser83 and Ser967 (Fujii *et al.*, 2004; Goldman *et al.*, 2004; Kim *et al.*, 2001). Therefore, we examined whether stimulation of TLR2 with MLP affected the phosphorylation state of these serine residues. MLP clearly induced phosphorylation of Ser83 residue in endogenous ASK1 at the concentration of $0.1 \mu\text{g ml}^{-1}$ in 293/TLR2-V5 cells (Fig. 6). However, the phosphorylated residue was dephosphorylated by stimulation with $1 \mu\text{g ml}^{-1}$ of MLP in a time-dependent fashion (Fig. 6). Additionally, stimulation with $10 \mu\text{g ml}^{-1}$ of MLP clearly induced phosphorylation of the residue at the earlier time points (5–15 min; data not shown), and then induced complete dephosphorylation of the residue at least 1 h after stimulation (Fig. 6). In contrast to these, MLP did not affect the phosphorylation state of Ser967 residue at all concentrations tested (Fig. 6).

Thus, it is likely that TLR2-mediated signaling has a potential to regulate the catalytic function of ASK1 via phosphorylation and dephosphorylation at the Ser83 residue in ASK1.

Discussion

In this study, we demonstrated that stimulation of TLR2 with microbial components, MLP or PGN, initiated activation of stress-activated MAPKs, which was accompanied by intracellular ROS generation (Figs 1 and 2). Several studies have already shown that stimulation of TLR2 with synthetic bacterial lipopeptides induced ROS generation, or oxidative burst, that is thought to be mediated by the NAD(P)H oxidase system (Aliprantis *et al.*, 1999 and 2001). In relation to this, a recent study has indeed demonstrated that the NAD(P)H oxidase member Nox4, which was highly expressed in HEK293 cells (Shiose *et al.*, 2001), was involved in TLR4-induced ROS generation (Park *et al.*, 2004). Elevated ROS are thought to serve as a second messenger to control a broad range of physiological and pathological processes, including cell proliferation, inflammation and apoptosis (Martindale and Holbrook, 2002; Torres and Forman, 2003; Ueda *et al.*, 2002; Yodoi *et al.*, 2001). It is now thought that ASK1 emerges as a major regulator of ROS-initiated signaling in response to various kinds of cellular stressors, including several ligand-receptor systems (Chen *et al.*, 2003; Takeda *et al.*, 2002; Yodoi *et al.*, 2001). ASK1 is present in cytosol as an inactive complex with thioredoxin (Trx), and then dissociate from Trx leading to active form after Trx oxidization by ROS (Takeda *et al.*, 2002; Yodoi *et al.*, 2001). ASK1 then forms a stable complex by interaction with TRAFs followed by activation of specific MAPKKs (Nishitoh *et al.*, 1998; Takeda *et al.*, 2002). Downstream of TLR2, ASK1 therefore appears to be activated by NAD(P)H oxidase system-induced ROS, and then mediates activation of MAPKs, especially sustained activation of p38 MAPK.

It is well researched that TLR2 recruits MyD88 as an adopter molecule to initiate subsequent signaling. In this study, we demonstrated that ASK1 participated in MLP- or

PGN-induced TLR2 signaling pathways. Although it remains unclear whether the ROS/ASK1-mediated responses are dependent on MyD88-mediated machineries, it is speculated that ASK *per se* is not absolutely indispensable for the initiation of TLR2 signaling, judging from the results that ASK1-KM did not abrogate TLR2-mediated activations of AP-1 and NF- κ B (Fig 4) and the machinery of ASK1 activation as described above. This speculation is also supported by the previous findings that ASK1-mediated MAPK cascades are inessential for the death receptor-induced proapoptotic signaling (Takeda *et al.*, 2002, Tobiume *et al.*, 2001). However, Takeda *et al* previously described that ASK1 appears to function as a multifunctional stress-sensing kinase to control cell survival and death in response to various kinds and strengths of stresses (Takeda *et al.*, 2002). Our present findings may support these multiple functions of ASK1 in microbial stimulus-initiated TLR2 signaling.

We demonstrated that ASK1-mediated activation of p38 MAPK downstream of TLR2 could regulate the transcriptional activity of both AP-1 and NF- κ B (Fig. 4). It is well known that AP-1 activity is tightly regulated by three MAPK members, p38 MAPK, JNK and ERK (Bradley and Poher, 2001; Dong *et al.*, 2002; Tanoue and Nishida, 2003). We examined the effects of SB203580 and SP600125 on TLR2-mediated AP-1 activity and expectedly found that AP-1 activation was blocked by both of these inhibitors (Fig. 4A). In addition, we also found that the MLP-induced NF- κ B activation could be partially blocked by SB203580 and, to a lesser extent, SP600125 (Fig. 4B). These are supported by several reports which have demonstrated that the activation of p38 MAPK and JNK can induce NF- κ B activation (Carter *et al.*, 1999; Schulze-Osthoff *et al.*, 1997; Vanden Berghe *et al.*, 1998). Therefore, it can be considered that ASK1-mediated p38 MAPK-dependent signaling cascades directly affect TLR2-mediated activation of both AP-1 and NF- κ B, which promotes cell survival and activation.

We further demonstrated that the ASK1-mediated MAPK activation could also affect the induction of TLR2-mediated apoptotic reactions (Fig. 5). This observation is also supported by the previous findings that ASK1 is involved in the regulation of the apoptotic pathways downstream of TNFR1 and Fas (Hatai *et al.*, 2000; Ichijo *et al.*, 1997; Takeda *et al.*, 2002). ASK1 could evidently affect TLR2-induced DNA fragmentation and caspase-3/7 activation, but weakly affected activation of caspase-8 and -9, mainly via p38 MAPK activation. Therefore, ASK1 appears to function upstream of caspase-3/7 in TLR2-mediated death signaling that may be originally triggered through MyD88 and FADD. These findings further suggest that ASK1 mainly modulates the execution of TLR2-mediated apoptotic reactions by regulating caspase-3/7, which are known as executioner caspases, but not by regulating initiator caspases. Indeed, ASK1 has been shown to affect pro- and anti-apoptotic molecules, such as Bcl-2, Bax and CIHA, which function upstream of the executioner caspases (Cho *et al.*, 2003; Takeda *et al.*, 2002; Yuan *et al.*, 2003).

The functions of ASK1 are regulated by multiple mechanisms. The prominent mechanisms by which the catalytic activity of ASK1 is regulated are thought to be protein-protein interactions. The major up-regulatory factors of ASK1, including TRAFs, Daxx, AIP1/DAB2IP, JSAP1/JIP3 and hD53L1, have already been reported (Chang *et al.*, 1998; Cho *et al.*, 2002; Matsuura *et al.*, 2002; Nishitoh *et al.*, 1998; Takeda *et al.*, 2002; Zhang *et al.*, 2004b; Zhang *et al.*, 2003). In contrast to this, Trx, 14-3-3 proteins, glutaredoxin, GSTM1-1, Hsp72 and Raf-1 were shown to be down-regulators (Chen *et al.*, 2001; Goldman *et al.*, 2004; Park *et al.*, 2002; Takeda *et al.*, 2002). The activation, oligomerization and protein-protein interactions of ASK1 are thought to be largely dependent on the phosphorylation at several Ser/Thr residues in ASK1. Indeed, phosphorylation at Thr838 residue in ASK1 is known to initiate the activation of ASK1

(Takeda *et al.*, 2002). On the other hand, the binding of 14-3-3 proteins to ASK1 is dependent on phosphorylation at Ser967 residue in ASK1, leading to inhibition of ASK1 activity (Goldman *et al.*, 2004; Zhang *et al.*, 2003). Furthermore, Akt/protein kinase B phosphorylates Ser83 residue in ASK1 to neutralize the catalytic functions of ASK1 (Kim *et al.*, 2001; Yuan *et al.*, 2003). Recently, the phosphorylation at Ser1034 residue in ASK1 was also reported to participate in the inhibition of ASK1 (Fujii *et al.*, 2004). In this study, we demonstrated that stimulation of TLR2 with a low dose of MLP induced phosphorylation at Ser83 residue in ASK1, whereas stimulation with a high dose of MLP induced dephosphorylation of the residue (Fig. 6). This observation indicates that TLR2 signaling regulates ASK1 activity, which may be due to the activity of Akt and an unknown protein phosphatase, and allusively shows the reason why ASK1 is only involved in sustained, but transient, activation of MAPKs (p38 MAPK in this study). In addition, this our finding supports the evidence that ASK1 senses strengths of TLR2-mediated stresses. Further study should be carried out to elucidate the extended regulatory mechanisms of ASK1, including oligomerization and protein-protein interactions, downstream of TLR2.

In Fig. 7, we showed schematic of ASK1 functions in TLR2 signaling pathways. Our observations suggest that ASK1 works as an important intermediate in microbial stimulus-induced TLR2 signaling through its regulatory effects on p38 MAPK, transcriptional factors and apoptotic reactions (Fig. 7). Originally, TLR2 is expressed on the surface of various types of cells to detect microbes (and/or their components) followed by initiation of MyD88-dependent innate immune reactions and apoptosis, whereas ASK1 is expressed in the cytosol of various types of cells to detect intracellular ROS following initiation of stress reactions, including the activation of stress-activated MAPKs and apoptosis. After ligation of

cognate PAMPs such as MLP and PGN with TLR2, ASK1 may function to control the MyD88-dependent divergent signaling pathways, which are largely dependent on the amount of microbes (and/or microbial components) and generated ROS. Therefore, our future study will focus on these regulatory mechanisms and the function of ASK1 in TLR2-mediated multiple cellular responses, innate immune responses and microbial infectious diseases.

Experimental procedures

Reagents, chemicals and antibodies (Abs)

PGN derived from *Staphylococcus aureus* was obtained from Fluka Production. Zymosan A was purchased from Sigma. Selective inhibitors for p38 MAPK (SB203580) and JNK (SP600125) were purchased from Calbiochem. All of mAbs to human MAPKs (phospho-specific p38, p38 α , phospho-specific JNK and JNK1/2) were obtained from BD Pharmingen. Abs to V5-epitope tag and HA-epitope tag were purchased from Invitrogen and Santa Cruz Biotechnology, Inc., respectively. Abs to human ASK1 and phospho-ASK1 (Ser83 and Ser967) were obtained from Cell Signaling Technology, Inc. An Ab to extracellular domain of human TLR2 was described previously (Fujita *et al.*, 2003).

All of the other chemicals were obtained from commercial sources and were of analytical or reagent grade.

Preparation of MLP

Mycoplasma fermentans ATCC 19989 was grown in PPLO broth supplemented with 20% (v/v) horse serum, 1% (w/v) yeast extract, 1% (w/v) D-glucose and penicillin G (1000 U ml⁻¹). When there was a fall in pH of 1 unit, the cells were collected by centrifugation at 15,000 g for 15 min, washed three times with PBS, and suspended in PBS. The cells were treated with Triton X-114 to extract MLP according to the method described previously (Into *et al.*, 2002c; Shibata *et al.*,

2000). MLP were dissolved in PBS containing 5% (v/v) DMSO at the concentration of 3 mg ml⁻¹ and stored at -80°C.

DNA expression vectors

The cDNA construct of C-terminal V5-epitope tagged human TLR2 cloned in the pEF6 vector was described previously (Into *et al.*, 2004). The construct containing HA-tagged human ASK1 cloned in the pcDNA3 vector was kindly provided by Prof. Hidenori Ichijo (Tokyo University, Japan), and kinase-inactive version of ASK1 (Lys709Met; ASK1-KM) (Ichijo *et al.*, 1997; Takeda *et al.*, 2002) was generated by using a Quick Change site directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Cell culture and transfection

HEK293 cells were obtained from ATCC and grown at 37°C and in 5% CO₂ in DMEM (Sigma) supplemented with 10% FBS (Sigma) and penicillin (100 U ml⁻¹) / streptomycin (100 µg ml⁻¹) (Sigma). A TLR2-V5 construct or a pEF6 empty vector was transfected into HEK293 cells using Metafectene lipofection reagent (Biontex Laboratories) according to the manufacturer's instructions. Stable transfectants were selected in the presence of blasticidin (50 µg ml⁻¹; Invitrogen), followed by immunoblot analysis to confirm the expression of TLR2-V5 using Abs to either TLR2 or V5-epitope. An HA-tagged ASK1-KM construct or a pcDNA3 empty vector was transfected into 293/TLR2-V5 cells using Metafectene lipofection reagent. Stable transfectants were selected in the presence of both G418 (800 µg ml⁻¹; Sigma) and blasticidin

(50 $\mu\text{g ml}^{-1}$), followed by immunoblot analysis to confirm the expression of ASK1-KM and TLR2-V5 using Abs to ASK1, HA-epitope and V5-epitope. Cells of the human promonocytic cell line THP-1 were obtained from Health Science Research Resources Bank and grown at 37°C and in 5% CO_2 in RPMI 1640 (Sigma) supplemented with 10% FBS and penicillin/streptomycin. An HA-tagged ASK1-KM construct or a pcDNA3 empty vector was transfected into THP-1 cells using Metafectene lipofection reagent. Stable transfectants were selected in the presence of G418 at the concentration of 800 $\mu\text{g ml}^{-1}$.

Luciferase reporter gene assay

The luciferase reporter gene assay in HEK293-derived transfectants was carried out essentially by the method described previously (Fujita *et al.*, 2003; Into *et al.*, 2004). Briefly, the cells were plated at 5×10^4 cells/well in poly-L-lysine-coated 24-well plates before DNA transfection. The cells were transiently transfected by Metafectene lipofection reagent with 50 ng of an NF- κ B reporter plasmid (pNF- κ B-Luk; Stratagene) or an AP-1 reporter plasmid (pAP-1-Luc; Stratagene) together with 5 ng of a construct directing expression of Renilla luciferase under the control of a constitutively active thymidine kinase promoter (pRL-TK; Promega). After 24 h of incubation, the cells were stimulated with MLP or PGN in media containing 1% FBS for 6 h. Then the cells were lysed and luciferase activity was measured by using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Results are expressed as means $\pm$ SD of three determinations.

Western blot analysis

HEK293 or THP-1 cell-derived transfectants were stimulated with or without MLP. Then the cells were collected and lysed in a buffer consisting of 20 mM Tris-hydrochloride (pH 7.2), 150 mM sodium chloride, 5 mM EDTA and 1% Nonident P-40 in the presence of protease inhibitors (Roche) at 4°C for 15 min followed by clarification by centrifugation at $12,000 \times g$ for 10 min. These cell lysates were diluted by an equal volume of SDS sample buffer consisting of 0.5 M Tris-hydrochloride (pH 7.2), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.05% bromophenol blue. Samples were boiled for 5 min and separated under reducing conditions on 10% SDS-PAGE gels and then transferred onto polyvinylidene fluoride membranes (Sigma). Membranes were blocked at room temperature for 1 h with 10% nonfat skim milk in PBS and then reacted with the Abs for 1 h. Immunoreactive bands were visualized by an ECL system (Amersham Pharmacia Biotech) after being treated with an Ab to HRP-conjugated anti-mouse or anti-rabbit IgG. All experiments were performed at least three times, and representative results are shown in the figures.

Detection of ROS

The generation of intracellular ROS was quantified fluorometrically using a cell-permeable oxidation-sensitive fluorescent probe, CM-H₂DCFDA (Molecular Probes). 293/TLR2-V5 cells (2.5×10^5 cells) were plated in 6-well plates and pretreated at 37°C with DMEM containing 5 μ M CM-H₂DCFDA for 30 min. The cells were gently washed once with DMEM and then stimulated with MLP or PGN in DMEM containing 2.5% FBS. After stimulation for 1 h, the cells were harvested, washed twice with DMEM, resuspended in DMEM, and analyzed on a

FACSCalibur flow cytometer (BD Biosciences). Dead cells and debris were excluded from the analysis by electronic gating of forward and side scatters. Experiments were performed at least three times, and representative results are shown in the figures.

Determination of the activities of p38 MAPK and JNK by ELISA

HEK293-derived transfectants (2×10^4 cells) were seeded in poly-L-lysine-coated 96-well plates. The cells were stimulated with MLP and then assayed for determination of the activity of p38 MAPK or JNK using FACETM ELISA Kits (Active Motif) according to the manufacturer's instructions. In this study, we used a mAb to phospho-p38 MAPK (T180/Y182) or phospho-JNK (T183/Y185) as a primary Ab to detect activated p38 MAPK or JNK. Colorimetric reaction and crystal violet cell staining were measured by absorbance on a spectrophotometer at 450 nm and 595 nm, respectively. The measured OD₄₅₀ readings were corrected for cell number by dividing the OD₄₅₀ reading for a given well by the OD₅₉₅ reading for that well. Results were calculated as fold increase when the control cells were taken as 1 and are expressed as means $\pm$ SD of three determinations.

Cytotoxicity assay

Cytocidal activity of MLP, PGN and zymosan in THP-1 cells was assessed essentially by the method described previously (Into *et al.*, 2004). The caspase inhibitors Ac-IETD-CHO and Ac-DMQD-CHO were obtained from Calbiochem. Results are expressed as means $\pm$ SD of three determinations.

Detection of cell apoptosis by ELISA

The generation of mono- and oligonucleosomes due to DNA fragmentation during the apoptotic process was detected using a Cell Death Detection ELISA kit (Roche). HEK293 cell-derived transfectants were seeded at 4×10^4 cells/well in poly-L-lysine-coated 24-well plates before stimulation. After stimulation with MLP or PGN for 24 h, the cells were lysed using lysis solution provided in the kit for 20 min on ice. The cell extracts were centrifuged for 10 min at $14,000 \times g$. The supernatants containing mono- and oligonucleosomes were equally diluted for all samples and assayed according to the manufacturer's instructions. Briefly, 96-well plates were coated with an anti-histone Ab. After blocking the wells using a buffer provided in the kit, samples were added and incubated for 90 min. Then an anti-DNA Ab conjugated with HRP was introduced. Colorimetric changes were developed by adding the substrate of peroxidase (ABTS), and optical density was measured at 405 nm with a spectrophotometer. The results are expressed as enrichment factor (the ratio of the OD readings from treated cells and the OD values from corresponding control cells). Results are expressed as means $\pm$ SD of three determinations.

Caspase activity assay

Caspase activity was determined using the Caspase-Glo assay (Promega). Briefly, HEK293-derived transfectants (1×10^4 cells) were seeded in poly-L-lysine-coated 96-well plates and stimulated with 10 μ g/ml of MLP or PGN for 24 h. Then either the caspase-3/7, -8 or

-9 substrate was added to the culture and incubated at room temperature in the dark for 30 minutes. Following incubation, luminescence was measured. The amount of luminescence detected as relative light units was proportional to each caspase activity. Results were calculated as fold increase in the case when the control cells were taken as 1 and are expressed as means $\pm$ SD of three determinations.

Statistical analysis

All values were evaluated by statistical analyses. For the comparison of MLP- or PGN-treated versus untreated cells or ASK1-KM-transfected versus mock-transfected cells, data were analyzed using Student's *t*-test. To test the effect of inhibitor treatment, ANOVA multiple-group analysis was used. Differences were considered to be statistically significant at the level of $P < 0.01$.

References

- Abrahams, V.M., Bole-Aldo, P., Kim, Y.M., Straszewski-Chavez, S.L., Chaiworapongsa, T., Romero, R., and Mor, G. (2004) Divergent trophoblast responses to bacterial products mediated by TLRs. *J Immunol* **173**: 4286-4296.
- Akira, S., and Takeda, K. (2004) Toll-like receptor signaling. *Nat Rev Immunol* **4**: 499-511.
- Aliprantis, A.O., Weiss, D.S., and Zychlinsky, A. (2001) Toll-like receptor-2 transduces signals for NF- κ B activation, apoptosis and reactive oxygen species production. *J Endotoxin Res* **7**: 287-291.
- Aliprantis, A.O., Yang, R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., *et al.* (1999) Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science* **285**: 736-739.
- Aliprantis, A.O., Yang, R.B., Weiss, D.S., Godowski, P., and Zychlinsky, A. (2000) The apoptotic signaling pathway activated by Toll-like receptor 2. *EMBO J* **19**: 3325-3336.
- Arbibe, L., Mira, J.P., Teusch, N., Kline, L., Guha, M., Mackman, N., *et al.* (2000) Toll-like receptor 2-mediated NF- κ B activation requires Rac1-dependent pathway. *Nat Immunol* **1**: 533-540.
- Bradley, J.R., and Pober, J.S. (2001) Tumor necrosis factor receptor-associated factors (TRAFs). *Oncogene* **20**: 6482-6491.
- Carter, A.B., Knudtson, K.L. Monick, M.M., and Hunninghake, G.W. (1999) The p38 mitogen-activated protein kinase is required for NF- κ B-dependent gene expression: the role of TATA-binding protein (TBP). *J Biol Chem* **274**: 30858-30863.
- Chang, H.Y., Nishitoh, H., Yang, X., Ichijo, H., and Baltimore, D. (1998) Activation of

apoptosis signal-regulating kinase 1 (ASK1) by the adaptor protein Daxx. *Science* **281**: 1860-1863.

Chen, J., Fujii, K., Zhang, L., Roberts, T., and Fu, H. (2001) Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. *Proc Nat Acad Sci USA* **98**: 7783-7788.

Chen, M.C., Hwang, M.J., Chou, Y.C., Chen, W.H., Cheng, G., Nakano, H., *et al.* (2003) The role of apoptosis signal-regulating kinase 1 in lymphotoxin- β receptor-mediated cell death. *J Biol Chem* **278**: 16073-16081.

Cho, S., Ko, H.M., Kim, J.M., Lee, J.A., Park, J.E., Jang, M.S., *et al.* (2002) Positive regulation of apoptosis signal-regulating kinase 1 by hD53L1. *J Biol Chem* **279**: 16050-16056.

Cho, S.G., Kim, J.W., Lee, Y.H., Hwang, H.S., Kim, M.S., Ryoo, K., *et al.* (2003) Identification of a novel antiapoptotic protein that antagonizes ASK1 and CAD activities. *J Cell Biol* **163**: 71-81.

Dong, C., Davis, R.J., and Flavell, R.A. (2002) MAP kinase in the immune responses. *Annu Rev Immunol* **20**: 55-72.

Fujii, K., Goldman, E.H., Park, H.R., Zhang, L., Chen, J., and Fu, H. (2004) Negative control of apoptosis signal-regulating kinase 1 through phosphorylation of Ser-1034. *Oncogene* **23**: 5099-5104.

Fujita, M., Into, T., Yasuda, M., Okusawa, T., Hamahira, S., Kuroki, Y., *et al.* (2003) Involvement of leucine residues at positions 107, 112, and 115 in a leucine-rich repeat motif of human Toll-like receptor 2 in the recognition of diacylated lipoproteins and lipopeptides and *Staphylococcus aureus* peptidoglycans. *J Immunol* **171**: 3675-3683.

Gohda, J., Matsumura, T., and Inoue, J. (2004) TNFR-associated factor (TRAF) 6 is essential

- for MyD88-dependent pathway but not Toll/IL-1 receptor domain-containing adaptor-inducing IFN- β (TRIF)-dependent pathway in TLR signaling. *J Immunol* **173**: 2913-2917.
- Goldman, E.H., Chen, L., and Fu, H. (2004) Activation of apoptosis signal-regulating kinase 1 by reactive oxygen species through dephosphorylation at serine 967 and 14-3-3 dissociation. *J Biol Chem* **279**: 10442-10449.
- Hatai, T., Matsuzawa, A. Inoshita, S., Mochida, Y., Kuroda, T., Sakamaki, K., *et al.* (2000) Execution of apoptosis signal-regulating kinase 1 (ASK1)-induced apoptosis by the mitochondria-dependent caspase activation. *J Biol Chem* **275**: 26576-26581.
- Horng, T., Barton, G.M., Flavell, R.A., and Medzhitov, R. (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* **420**: 329-333.
- Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., *et al.* (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **275**: 90-94.
- Into, T., Fujita, M., Okusawa, T., Hasebe, A., Morita, M., and Shibata, K. (2002a) Synergic effects of mycoplasmal lipopeptides and extracellular ATP on activation of macrophages. *Infect Immun* **70**: 3586-3591.
- Into, T., Kiura, K., Yasuda, M., Kataoka, H., Inoue, N., Hasesbe, A., *et al.* (2004) Stimulation of human Toll-like receptor (TLR) 2 and TLR6 with membrane lipoproteins of *Mycoplasma fermentans* induce apoptotic cell death after NF- κ B activation. *Cell Microbiol* **6**: 187-199.
- Into, T., Nodasaka, Y., Hasebe, A., Okuzawa, T., Nakamura, J., Ohata, N., and Shibata, K. (2002b) Mycoplasmal lipoproteins induce caspases- and Toll-like receptor 2-mediated cell death in lymphocytes and monocytes. *Microbiol Immunol* **46**: 265-276.
- Into, T., Okada, K., Inoue, N., Yasuda, M., and Shibata, K. (2002c) Extracellular ATP regulates

cell death of lymphocytes and monocytes induced by membrane-bound lipoproteins of *Mycoplasma fermentans* and *Mycoplasma salivarium*. *Microbiol Immunol* **46**: 667-675.

Janeway, C.A. Jr, and Medzhitov, R. (1999) Lipoproteins take their Toll on the host. *Curr Biol* **9**: R879-882.

Janssens, S., and Beyaert, R. (2003) Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol Cell* **11**: 293-302.

Kim, A.H., Khursigara, G., Sun, X., Franke, T.F., and Chao, M.V. (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Biol* **21**: 893-901.

Kirschning, C.J., and Schumann, R.R. (2002) TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr Top Microbiol Immunol* **270**: 121-144.

Mansell, A., Brint, E., Gould, J.A., O'Neill, L.A., and Hertzog, P.J. (2004) Mal interacts with tumor necrosis factor-associated factor (TRAF)-6 to mediate NF- κ B activation by Toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* **279**: 37227-37230.

Martindale, J.L., and Holbrook, N.J. (2002) Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol* **192**: 1-15.

Matsuura, H., Nishitoh, H., Takeda, K., Matsuzawa, A., Amagasa, T., Ito, M., *et al.* (2002) Phosphorylation-dependent scaffolding role of JSAP1/JIP3 in the ASK1-JNK signaling pathway. A new mode of regulation of the MAP kinase cascade. *J Biol Chem* **277**: 40703-40709.

Medzhitov, R., Preston-Hurlburt P., and Janeway C.A. Jr. (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* **388**: 394-397.

Ninomiya-tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) The kinase TAK1 can activate the NIK-I κ B as well as the MAP kinase cascade in the IL-1

- signalling pathway. *Nature* **398**: 252-256.
- Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., *et al.* (1998) ASK1 is essential for JNK/SAPK activation by TRAF2. *Mol Cell* **2**: 389-395.
- O'Neill, L.A., Dunne, A., Edjeback, M., Gray, P., Jefferies, C., and Wietek, C. (2003) Mal and MyD88: adaptor proteins involved in signal transduction by Toll-like receptors. *J Endotoxin Res* **9**: 55-59.
- Park, H.S., Cho, S.G., Kim, C.K., Hwang, H.S., Noh, K.T., Kim, M.S., *et al.* (2002) Heat shock protein Hsp72 is a negative regulator of apoptosis signal-regulating kinase 1. *Mol Cell Biol* **22**: 7721-7730.
- Park, H.S., Jung, H.Y., Park, E.Y., Kim, J., Lee, W.J., and Bae, Y.S. (2004) Direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF- κ B. *J Immunol* **173**: 3589-3593.
- Schulze-Osthoff, K., Ferrari, D., Riehemann, K., and Wesselborg, S. (1997) Regulation of NF- κ B activation by MAP kinase cascades. *Immunobiology* **198**: 35-49.
- Shibata, K., Hasebe, A., Into, T., Yamada, M., and Watanabe, T. (2000) The N-terminal lipopeptide of 44-kDa membrane-bound lipoprotein of *Mycoplasma salivarium* is responsible for the expression of intercellular adhesion molecule-1 on the cell surface of normal gingival fibroblasts. *J Immunol* **165**: 6538-6544.
- Shiose, A., Kuroda, J., Tsuruya, K., Hirai, M., Hirakata, H., Naito, S., *et al.* (2001) A novel superoxide-producing NAD(P)H oxidase in kidney. *J Biol Chem* **276**: 1417-1423.
- Shuto, T., Xu, H., Wang, B., Han, J., Kai, H., Gu, X.X., *et al.* 2001. Activation of NF- κ B by nontypeable *Hemophilus influenzae* is mediated by Toll-like receptor 2-TAK1-dependent NIK-IKK α / β -I κ B α and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells. *Proc*

Nat Acad Sci USA **98**: 8774-8779.

Strassheim, D., Asehnoune, K., Park, J.S., Kim, J.Y., He, Q., Richard, D., *et al.* (2004)

Phosphoinositide 3-kinase and Akt occupy central roles in inflammatory responses of Toll-like receptor 2-stimulated neutrophils. *J Immunol* **172**: 5727-5733.

Suzuki, N., Suzuki, S., and Yeh, W.C. (2002) IRAK-4 as the central TIR signaling mediator in innate immunity. *Trends Immunol* **23**: 503-506.

Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K., *et al.* (2000) TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol Cell* **5**: 649-658.

Takeda, K., Kaisho, T. and Akira, T. (2003) Toll-like receptors. *Annu Rev Immunol* **21**: 335-376.

Takeda, K., Matsuzawa, A., Nishitoh, H., and Ichijo, H. (2002) Roles of MAPKKK ASK1 in stress-induced cell death. *Cell Struct Funct* **28**: 23-29.

Tanoue, T., and Nishida, E. (2003) Molecular recognitions in the MAP kinase cascades. *Cell Signal* **15**: 455-462.

Teusch, N., Lombardo, E., Eddleston, J., and Knaus, U.G. (2004) The low molecular weight GTPase RhoA and atypical protein kinase C ζ are required for TLR2-mediated gene transcription. *J Immunol* **173**: 507-514.

Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., *et al.* (2001) ASK1 is required for sustained activation of JNK/p38 MAP kinase and apoptosis. *EMBO Rep* **2**: 222-228.

Torres, M., and Forman, H.J. (2003) Redox signaling and the MAP kinase pathways. *Biofactors* **17**: 287-296.

Ueda, S., Masutani, H., Nakamura, H., Tanaka, T., Ueno, M., and Yodoi J. (2002) Redox

- control of cell death. *Antioxid Redox Signal* **4**: 405-414.
- Vanden Berghe, W., Plaisance, S., Boone, E., Bosscher, K., Schmitz, M.L., Fiers, W., and Haegeman, G. (1998) p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor- κ B p65 transactivation mediated by tumor necrosis factor. *J Biol Chem* **273**: 3285-3290.
- Yamamoto, M., Sato, S., Hemmmi, H., Sanjo, H., Uematsu, S., Kaisho, T., *et al.* (2002) Essential roles for TIRAP in activation of the signaling cascade shared by TLR2 and TLR4. *Nature* **420**: 324-329.
- Yodoi, J., Masutani, H., and Nakamura H. (2001) Redox regulation by the human thioredoxin system. *Biofactors* **15**: 107-111.
- Yuan, Z.Q., Feldman, R.I. Sussman, G.E., Coppola, D. Nicosia, S.V., and Cheng, J.Q. (2003) AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance. *J Biol Chem* **278**: 23432-23440.
- Zhang, D., Zhang, G, Hayden, M.S., Greenblatt, M.B., Bussey, C., Flavell, R.A., and Ghosh, S. (2004a) A Toll-like receptor that prevents infection by uropathogenic bacteria. *Science* **303**:1522-1526.
- Zhang, H., Zhang, R., Luo, Y., D'Alessio, A., Pober, J.S., and Min, W. (2004b) AIP/DA2IP, a novel member of the Ras-GAP family, transduces TRAF2-induced ASK1-JNK activation. *J Biol Chem* **279**: 44955-44965.
- Zhang, R., He, X., Liu, W., Lu, M., Hsieh, J.T., and Min, W. (2003) AIP1 mediates TNF- α -induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3. *J Clin Invest* **111**: 1933-1943.

Figure legends

Fig. 1. TLR2-mediated activation of stress-activated MAPKs induced by MLP and PGN.

A and B. 293/pEF6 and 293/TLR2-V5 cells were transiently transfected with an AP-1 (A) or an NF- κ B (B)-driven luciferase reporter construct. Then the cells were stimulated for 6 h with MLP, PGN or zymosan at the concentrations indicated. Cells were lysed and assayed for luciferase activities. Results, expressed as the means $\pm$ SD of values for triplicate wells, are representative of three separate experiments.

C. 293/TLR2-V5 cells were stimulated with MLP at the indicated concentrations for the indicated period. Then the cells were lysed and assayed for the phosphorylation states of p38 MAPK and JNK1/2 by Western blotting using phosphorylation-specific Abs (p-p38 and p-JNK1/2). Immunoblot analyses for phosphorylation-independent MAPKs (p38 α and JNK1/2) were also carried out as loading controls. The result shown is representative for three independent experiments.

Fig. 2. TLR2-mediated generation of ROS induced by MLP and PGN. An oxidation-sensitive fluorescence probe, CM-H₂DCFDA, was loaded into 293/TLR2-V5 cells. Then the cells were stimulated for 1 h with MLP (A) or PGN (B) at the indicated concentrations and assayed for flow cytometry. The shaded histogram represents the cells cultured without stimulators. Representative results of three separate experiments are shown.

Fig. 3. Involvement of ASK1 in TLR2-mediated stress-activated MAPK activation.

A. Parental 293, 293/TLR2-V5/pcDNA3 and 293/TLR2-V5/ASK1-KM cells were prepared by

the method described in “Materials and Methods”. Then the cells (1×10^6 cells/sample) were analyzed for the expression of endogenous ASK1, HA-tagged ASK1-KM and TLR2-V5 using an Ab to ASK1, HA-epitope and V5-epitope, respectively, by Western blotting (WB).

B and C. 293/TLR2-V5/pcDNA3 and 293/TLR2-V5/ASK1-KM cells were stimulated with MLP at the indicated concentrations for the indicated period. MLP-induced phosphorylation of p38 MAPK (B) and JNK (C) was determined by an ELISA method. Results are expressed as the means $\pm$ SD of values for triplicate wells. See “Experimental procedures” for details. All values were analyzed for statistical significance using Student’s *t*-test by comparison of ASK1-KM versus pcDNA3 in the respective periods. Differences were considered at the level of $P < 0.01$.

D. THP-1/pcDNA3 and THP-1/ASK1-KM cells (1×10^6 cells/sample) were stimulated with $10 \mu\text{g ml}^{-1}$ of MLP for the indicated period. Then the cells were lysed and assayed for the phosphorylation states of p38 MAPK and JNK1/2 by Western blotting using phosphorylation-specific Abs (p-p38 and p-JNK1/2). The result shown is representative for three independent experiments.

Fig. 4. Involvement of ASK1 in activation of AP-1 and NF- κ B induced MLP and PGN.

293/TLR2-V5/pcDNA3 cells and 293/TLR2-V5/ASK1-KM cells were transiently transfected with an AP-1 (A) or an NF- κ B (B)-driven luciferase reporter construct. Then the cells were stimulated for 6 h with MLP or PGN at the indicated concentrations. In the case of using MAPK inhibitors, 293/TLR2-V5/pcDNA3 cells were pretreated for 1 h with $25 \mu\text{M}$ of SB203580 or SP600125 and then stimulated with or without $10 \mu\text{g ml}^{-1}$ of MLP. The cells were lysed and assayed for luciferase activities. Results, expressed as the means $\pm$ SD of values for triplicate wells, are representative of three separate experiments. $*P < 0.01$, pcDNA3 versus ASK1-KM

(Student's *t*-test). ***P* < 0.01, versus inhibitor untreated (one-way AVOVA).

Fig. 5. Involvement of ASK1 in TLR2-mediated cell death.

A. THP-1 cells (0.5×10^5 cells) were pretreated for 1 h with $5 \mu\text{g ml}^{-1}$ isotype control IgG, $5 \mu\text{g ml}^{-1}$ TL2.1 antibody, the caspase-8 inhibitor Ac-IETD-CHO ($20 \mu\text{M}$) or the caspase-3 inhibitor Ac-DMQD-CHO ($20 \mu\text{M}$). Then the cells were incubated with $10 \mu\text{g ml}^{-1}$ MLP, $10 \mu\text{g ml}^{-1}$ PGN or $100 \mu\text{g ml}^{-1}$ zymosan. After 12 h of stimulation, the culture supernatant was assayed for lactate dehydrogenase (LDH) release. Results, expressed as the means $\pm$ SD of values for triplicate wells, are representative of three separate experiments. **P* < 0.01, versus untreated cells (one-way AVOVA).

B. 293/TLR2-V5 cells were stimulated for 24 h with MLP or PGN at the indicated concentrations. Then the cells were lysed and assessed by an ELISA method that detected DNA fragmentation. The enrichment factor 1 represents approximately 6% of apoptotic cells in our experiments. Results, expressed as the means $\pm$ SD of values for triplicate wells, are representative of three separate experiments.

C, D, E and F. 293/TLR2-V5/pcDNA3 and 293/TLR2-V5/ASK1-KM cells were stimulated for 24 h with MLP or PGN at the indicated concentrations. In the case of using MAPK inhibitors, 293/TLR2-V5/pcDNA3 cells were pretreated for 1 h with $25 \mu\text{M}$ of SB203580 or SP600125, and then stimulated with or without $10 \mu\text{g ml}^{-1}$ of MLP. Then the cells were lysed and assessed by an apoptosis ELISA (C). The cells were also assayed for activity of caspase-8 (D), caspase-9 (E) or caspase-3/7 (F). Results, expressed as the means $\pm$ SD of values for triplicate wells, are representative of three separate experiments. **P* < 0.01, pcDNA3 versus ASK1-KM (Student's *t*-test). ***P* < 0.01, versus inhibitor untreated (one-way AVOVA).

Fig. 6. Effect of stimulation of TLR2 with MLP on the phosphorylation of serine residues in ASK1. 293/TLR2-V5 cells (1×10^7 cells/sample) were prepared and stimulated with MLP at the indicated concentrations for the indicated period. The phosphorylated serine residues (Ser83 and Ser967) in ASK1 (p-ASK1) were examined by Western blotting. Immunoblot analysis for phosphorylation-independent endogenous ASK1 was also carried out as a loading control. The result shown is representative for three independent experiments.

Fig. 7. Schematic of MLP- or PGN-induced TLR2 signaling pathways. See the text for details.

Acknowledgements

We would like express our hearty thanks to Prof. Hidenori Ichijo, Tokyo University, Japan for providing an ASK1-HA construct. This work was supported by Grants-in-Aid for Science Research (no. B(2)15390549, 16791102 and B17390498) provided by the Ministry of Education, Culture, Sports, Science and Technology, Japan and by a grant from the Akiyama Foundation.

Fig. 1

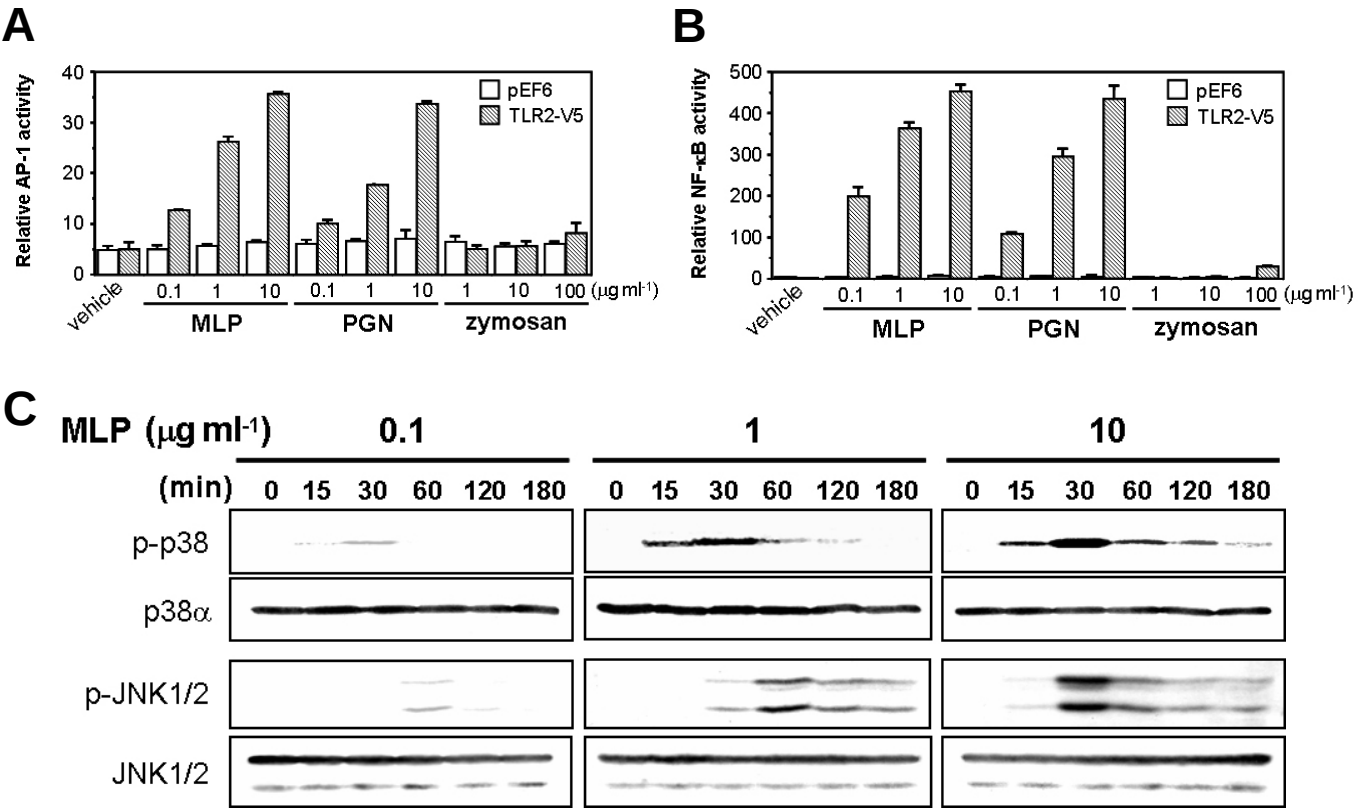


Fig.1

Fig. 2

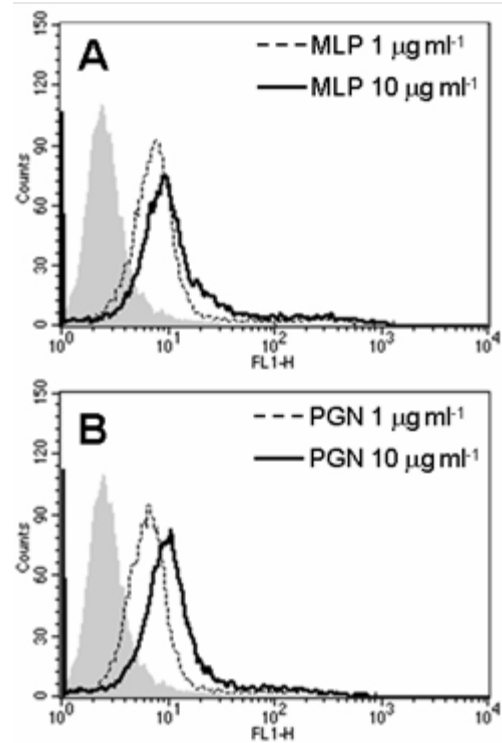


Fig. 3

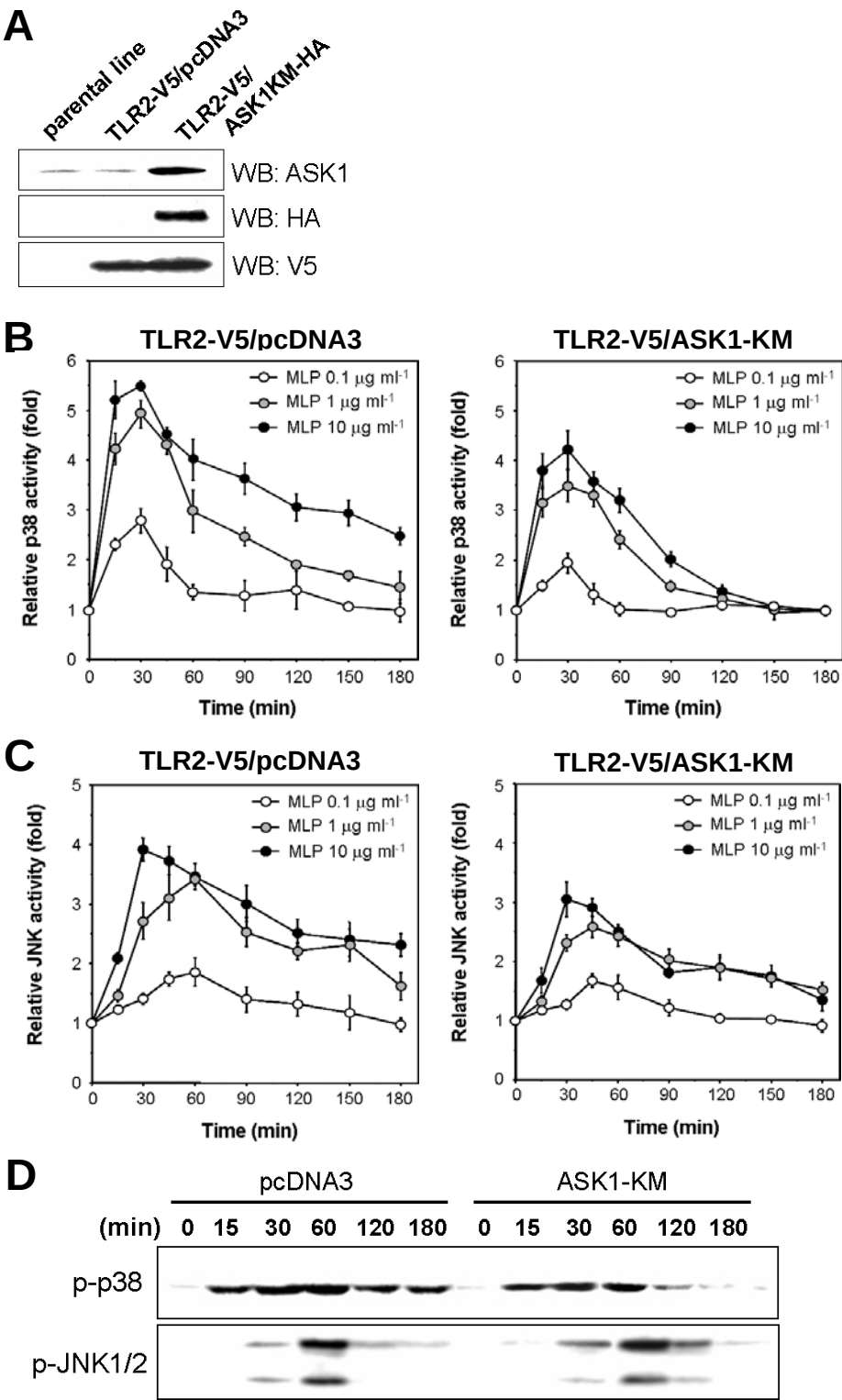


Fig. 4

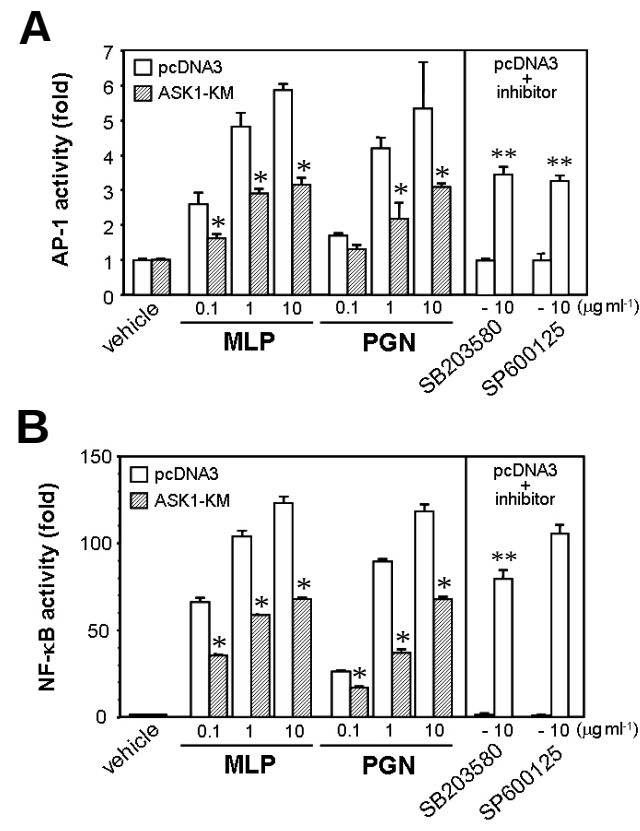


Fig. 5

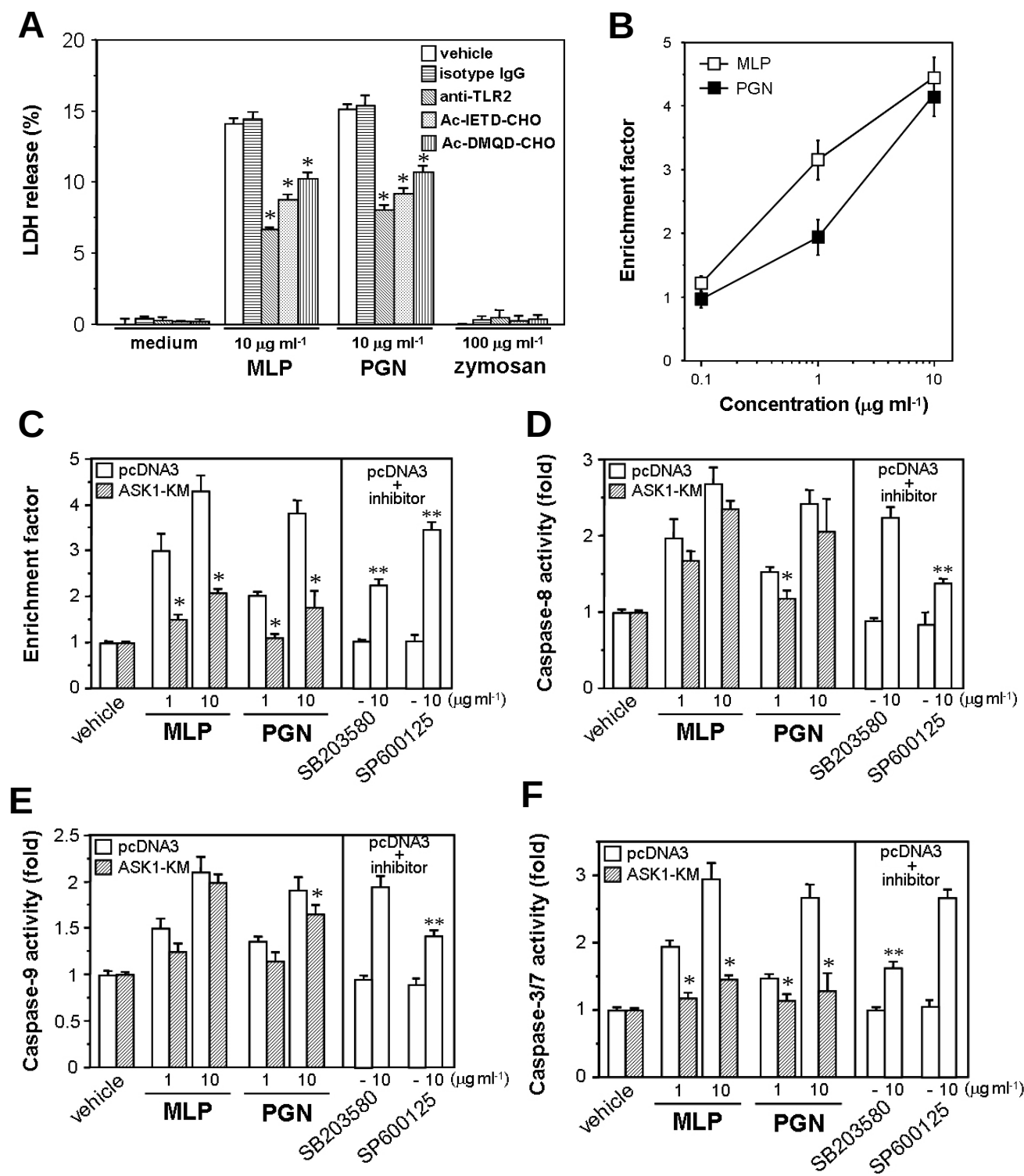


Fig. 6

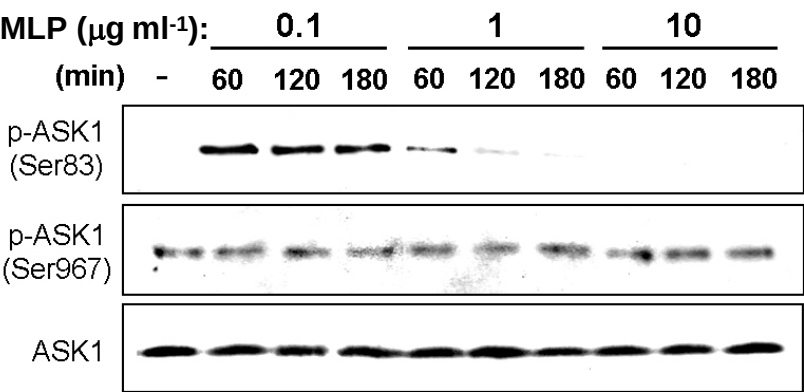


Fig. 7

