

A variety of microbial components induce tolerance to lipopolysaccharide by differentially affecting MyD88-dependent and -independent pathways

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Abstract

Exposure of macrophages to lipopolysaccharide (LPS) induces a hypo-responsive state to a second challenge with LPS that is termed LPS tolerance. LPS tolerance is also induced by pre-exposure to lipopeptides and lipoteichoic acid, which trigger Toll-like receptor (TLR) 2-mediated signaling. LPS signaling involves at least two pathways: a MyD88-dependent cascade that is essential for production of inflammatory cytokines and a MyD88-independent cascade that mediates the expression of IFN-inducible genes. We analyzed the induction of LPS tolerance by several microbial components in mouse peritoneal macrophages. Pre-exposure to LPS led to impaired activation of both the pathways. In contrast, mycoplasmal lipopeptides did not affect the MyD88-independent pathway, but impaired the MyD88-dependent signaling by inhibiting LPS-mediated activation of IL-1 receptor-associated kinase (IRAK) 1. The induction of LPS tolerance by recently identified TLR ligands was analyzed. Pretreatment with double-stranded RNA, which triggers the activation of TLR3, led to defective activation of the MyD88-independent, but not the MyD88-dependent, pathway. Imidazoquinoline compounds, which are recognized by TLR7, had no effect on the MyD88-independent pathway, but inhibited LPS-induced activation of MyD88-dependent signaling through down-regulation of IRAK1 expression. Thus, each microbial component induced LPS tolerance in macrophages.

Introduction

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is a potent activator of innate immune cells, and induces fatal endotoxin shock syndrome with exaggerated production of inflammatory cytokines in experimental animals and humans. In the clinic, however, endotoxin shock is not common in patients suffering from Gram-negative sepsis. This may be partly attributable to the induction of LPS tolerance, a phenomenon whereby pre-exposure to LPS causes a reduced response to subsequent challenge with LPS (1,2). Thus, LPS tolerance is considered a

host response to prevent excessive inflammation and shock syndrome. However, it can also be a major problem in the treatment of patients with Gram-negative sepsis. This is because some patients that recover from septic shock become tolerant to LPS and have a severely reduced host defense response to opportunistic infections leading to high mortality (3). Therefore, the molecular mechanism for LPS tolerance has long been investigated.

LPS tolerance was first demonstrated in experimental animals (4). Subsequent studies showed that it also occurs

in macrophages/monocytes (5). LPS tolerance in macrophages/monocytes has been investigated extensively and shown to be characterized by a decline in the production of inflammatory cytokines including tumor necrosis factor (TNF)- α , IL-6 and IL-12 (5–7), and alterations to the LPS-induced activation of signaling cascades including protein kinase C, phosphatidylinositol-3 kinase, mitogen-activated protein (MAP) kinases and I κ B kinases (8–12). Accumulation of the p50 subunit of NF- κ B, which has no transactivating activity, has also been reported (13,14). However, the precise mechanism responsible for LPS tolerance remains unclear. Furthermore, recent studies have indicated that LPS tolerance in macrophages is induced by not only LPS, but also several microbial components such as lipopeptides, CpG DNA and lipoteichoic acid (15–19).

Toll-like receptors (TLR) have been shown to be essential for the recognition and discrimination of microbial components. TLR4 recognizes LPS, whereas TLR2 recognizes PGN and lipopeptides (20,21). Activation of TLR by microbial components facilitates recruitment of a serine/threonine kinase IL-1 receptor-associated kinase (IRAK) 1 to TLR via the adaptor molecule MyD88. Activated IRAK1 associates with TNF receptor-associated factor (TRAF) 6, thereby leading to activation of the c-Jun N-terminal kinase (JNK)/p38 MAP kinases and the Rel family of transcription factor NF- κ B. An essential role for MyD88 in the signaling via TLR has been demonstrated in MyD88-deficient mice. MyD88-deficient mice do not produce inflammatory cytokines in response to a variety of microbial components including LPS (TLR4 ligand), lipopeptides (TLR2 ligand), flagellin (TLR5 ligand) and CpG DNA (TLR9 ligand) (22–27). However, stimulation of MyD88-deficient macrophages with LPS caused the activation of JNK/p38 and NF- κ B, although these cells did not show any response to lipopeptides and CpG DNA (24). These findings imply that LPS-mediated signaling consists of at least two pathways; MyD88-dependent and -independent pathways. MyD88-dependent LPS signaling is essential for the production of inflammatory cytokines. MyD88-independent LPS signaling has recently been shown to induce the maturation of dendritic cells and induction of IFN-inducible genes (28,29).

The involvement of TLR in the induction of LPS tolerance has recently been demonstrated. Down-regulation of the surface expression of the TLR4-MD2 complex and TLR4 has been shown to cause LPS-induced LPS tolerance in mouse peritoneal macrophages and THP-1 human monocytic cell lines respectively (30,31). Changes to the TLR-mediated signaling pathways have also been reported in LPS-tolerized cells. LPS pretreatment induced reduced expression of IRAK1 in THP-1 cells (32). LPS tolerance induced by lipopeptides and LTA, both of which are recognized by TLR2, has been found to occur through a distinct mechanism from LPS-induced LPS tolerance (15,19). These findings imply that LPS tolerance was differentially induced by each TLR ligand.

In this study, we closely compared the LPS tolerance induced by LPS and lipopeptides derived from *Mycoplasma fermentans* [macrophage-activating lipopeptide-2 kDa (MALP-2)]. LPS-induced tolerance blocked both the MyD88-dependent and -independent pathways, possibly due to down-regulation of the surface expression of the TLR4-MD2 complex. In contrast, MALP-2 pretreatment impaired the

activation of the MyD88-dependent pathway by inhibiting IRAK1 activation, but it did not affect the MyD88-independent pathway. We further analyzed the induction of LPS tolerance by newly identified TLR ligands. Double-stranded RNA, which has recently been indicated to be recognized by TLR3, affected the MyD88-independent, but not MyD88-dependent, pathway. A low mol. wt synthetic compound, R-848, which is recognized by TLR7, affected the activation of the MyD88-dependent pathway through down-modulation of IRAK1 expression. Thus, LPS tolerance is differentially induced by several microbial components, which may explain the complicated nature of LPS tolerance.

Methods

Cells

Peritoneal macrophages were isolated from C57BL/6J mice, ICR mice (SLC, Shizuoka, Japan) or MyD88-deficient mice. Briefly, mice were injected i.p. with 2 ml of 4% thioglycollate. After 3 days, peritoneal exudate cells were isolated by washing the peritoneal cavity with ice-cold HBSS. These cells were incubated for 2 h and adherent cells were used as peritoneal macrophages. RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FBS (Life Technologies), 2-mercaptoethanol (100 μ M), penicillin (5 U/ml) and streptomycin (50 ng/ml) was used as the culture medium.

Reagents and antibodies

LPS from *Salmonella minnesota* Re595 prepared by phenol-chloroform-petroleum ether extraction procedure was purchased from Sigma (St Louis, MO). MALP-2 and R-848 were synthesized and purified as described previously (23,33). Poly(I:C) was provided by Yamasa (Tokyo, Japan).

Rabbit anti-IRAK1 polyclonal antibody was raised against a peptide corresponding to amino acids 693–710 of mouse IRAK1. Anti-IFN-regulatory factor (IRF)-3 antibody was as previously described (34). Rabbit anti-I κ B α , anti-ERK1/2 and anti-TRAF6 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-ERK1/2 antibody was purchased from New England Biolabs (Beverly, MA). Rabbit anti-MyD88 antibody was purchased from ProSci (Poway, CA). Rat anti-Tollip antibody was purchased from Alexis Biochemicals (San Diego, CA).

Northern blot analysis

Peritoneal macrophages (1×10^7) were pre-incubated with 100 ng/ml of *S. minnesota* Re595 LPS, 3 ng/ml of MALP-2, 100 nM R-848 or 100 μ g/ml of poly(I:C) for 24 h and washed twice with the culture medium. At 4 h after a second stimulation, total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD). The RNA (10 μ g) was electrophoresed, transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Uppsala, Sweden) and hybridized with a cDNA probe specific for that particular molecule.

Measurement of cytokine concentration

Peritoneal macrophages (1×10^5) were treated with the indicated stimulus for 24 h and washed twice with culture

medium. Then, cells were re-stimulated for 24 h. Concentrations of TNF- α in the culture supernatant were measured by ELISA according to the manufacturer's instructions (Genzyme Techne, Minneapolis, MN).

Western blot analysis

Cells were lysed in a lysis buffer containing 1.0% NP-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA and protease inhibitor cocktail (Roche, Mannheim, Germany). The cell lysates were dissolved by SDS-PAGE and transferred onto a PVDF membrane (BioRad, Hercules, CA). The membrane was incubated with the relevant antibodies and visualized with an enhanced chemiluminescence system (NEN Life Science Product, Boston, MA).

In vitro kinase assay

IRAK1 was immunoprecipitated by rabbit anti-IRAK1 polyclonal antibody, which was kindly provided by Hayashibara (Okayama, Japan) from prepared cell lysates. Immunoprecipitants were subjected to an *in vitro* kinase assay as described previously (24).

Native PAGE

Native PAGE was performed as described previously (34). Briefly, a 7.5% gel was pre-run with 25 mM Tris and 192 mM glycine with or without 1% deoxycholate in the upper and lower chamber respectively for 30 min at 40 mA. Samples in the native sample buffer (62.5 mM Tris-HCl, pH 6.8, 15% glycerol) were applied to the gel and electrophoresed for 60 min at 25 mA. This was followed by Western blotting using anti-IRF-3 antibody.

Results

IFN-inducible genes were induced in response to LPS in MALP-2 pretreated macrophages

We previously showed that MALP-2 derived from *M. fermentans* induced LPS tolerance in terms of TNF- α production (15). We first analyzed whether MALP-2-induced LPS tolerance was observed on the induction of various inflammatory mediators. Thioglycollate-elicited mouse peritoneal macrophages were pretreated with LPS or MALP-2 for 24 h and then re-stimulated with LPS or MALP-2 for 4 h. LPS, used in this paper, could not induce any inflammatory responses on TLR4-deficient mice and cells, even if used at high concentration (35). Total RNA was isolated from these cells and subjected to Northern blot analysis (Fig. 1A). Consistent with the previous study, the mRNA expression of TNF- α was induced in response to LPS and MALP-2 in non-pretreated cells. In LPS-pretreated cells, MALP-2-induced, but not LPS-induced, TNF- α expression was observed. In MALP-2-pretreated cells, neither LPS nor MALP-2 induced TNF- α mRNA expression. We also hybridized the same membrane with several cDNA probes such as IL-1 β , IL-12p40 and COX-2. The expression patterns of these genes in response to LPS and MALP-2 were the same as those for TNF- α . Thus, MALP-2 pretreatment induced LPS tolerance in terms of the expression of not only TNF- α , but several other inflammatory mediators.

We recently showed that there are two cascades in LPS signaling. One is MyD88 dependent, and essential for the LPS-induced expression of genes for inflammatory mediators such as TNF- α , IL-1 β , IL-12p40 and COX-2. The other is MyD88 independent and responsible for the LPS-induced expression of IFN-inducible genes (29). We next analyzed the LPS-induced expression of IFN-inducible genes such as IP-10, GARG16 and Best5 (Fig. 1B). MyD88-independent induction of IFN-inducible genes has been observed in LPS signaling, but not in MALP-2 signaling. Indeed, LPS induced the expression of these genes, whereas MALP-2 did not, in non-pretreated cells. In LPS-pretreated cells, LPS induction of the IFN-inducible genes was not observed. However, in MALP-2-pretreated cells, LPS stimulation induced expression of the IFN-inducible genes to a similar extent to that in non-pretreated cells. These results indicate that LPS pretreatment led to the impaired activation of both MyD88-dependent and -independent pathways. In contrast, MALP-2 pretreatment affected the MyD88-dependent, but not MyD88-independent, pathway.

LPS-induced activation of IRF-3, NF- κ B and ERK1/2 in MALP-2-pretreated macrophages

IRF-3 has been shown to be activated in response to LPS in MyD88-deficient macrophages, demonstrating the involvement of IRF-3 in the MyD88-independent signaling pathway (29). Therefore, we analyzed LPS-induced activation of IRF-3 in LPS- or MALP-2-pretreated macrophages (Fig. 2A). We assessed IRF-3 activation by a recently developed assay using native PAGE (34). In non-pretreated cells, LPS stimulation led to the generation of a slow migrating band that corresponds to the asymmetric homodimer of the phosphorylated IRF-3 (arrowhead). In LPS-pretreated cells, LPS stimulation did not induce the generation of the IRF-3 homodimer. In contrast, LPS stimulation induced the homodimerization of IRF-3 in MALP-2-pretreated cells. Thus, LPS-induced IRF-3 activation was observed in MALP-2-pretreated cells similar to that in non-pretreated cells.

LPS-induced activation of NF- κ B, albeit delayed, has also been demonstrated in MyD88-deficient mice (24). We previously showed that the LPS-induced DNA binding activity of NF- κ B was severely reduced in MALP-2-pretreated cells (15). We speculated that LPS-induced NF- κ B activation was delayed in MALP-2-pretreated cells, in which LPS-induced activation of MyD88-independent signaling was observed. Therefore, macrophages were stimulated with LPS, harvested at various time points and analyzed for degradation of I κ B α (Fig. 2B). In non-pretreated cells, LPS-induced degradation of I κ B α was observed as early as 7.5 min after stimulation. In LPS-pretreated cells, I κ B α did not degrade in response to LPS at all. In MALP-2-pretreated cells, the expression of I κ B α was not reduced at 7.5 min of LPS stimulation; however, it was reduced at 15 min and more severely at 30 min. Thus, LPS-induced degradation of I κ B α was actually observed in MALP-2-pretreated cells, although the kinetics was delayed.

We also analyzed the LPS-induced activation of ERK1/2 in MALP-2-pretreated cells. Peritoneal macrophages pretreated with LPS or MALP-2 for 24 h were re-stimulated with LPS for 15 or 30 min and analyzed for phosphorylation of ERK1/2 by Western blotting (Fig. 2C). After 15 min of re-stimulation with

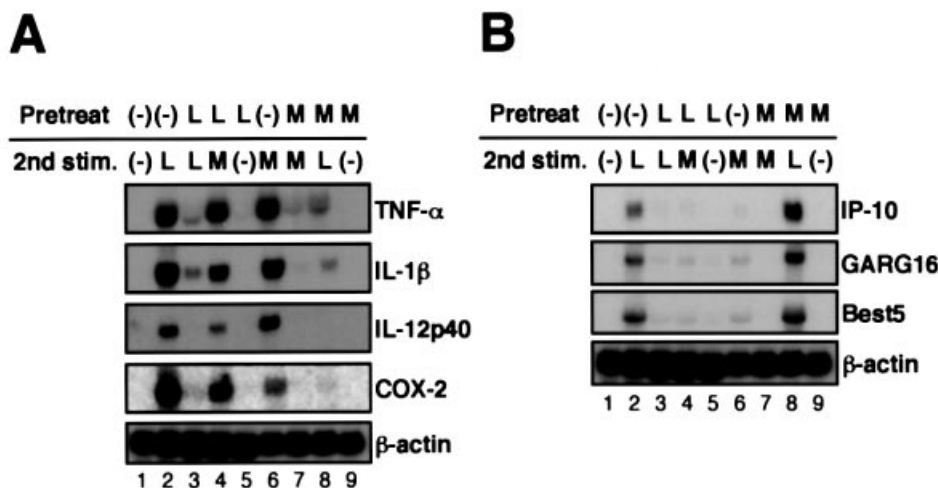


Fig. 1. IFN-inducible genes, but not cytokine genes, were induced by LPS stimulation in MALP-2-pretreated macrophages. Mouse peritoneal macrophages were pretreated with 100 ng/ml of LPS or 3 ng/ml of MALP-2 for 24 h. Cells were washed twice and then stimulated with 100 ng/ml of LPS or 3 ng/ml of MALP-2 for 4 h. Total RNA was extracted, and subjected to Northern blot analysis using specific cDNA probes for inflammatory cytokine genes (A) and IFN-inducible genes (B). The results are representative of three independent experiments that had similar results. L, LPS; M, MALP-2.

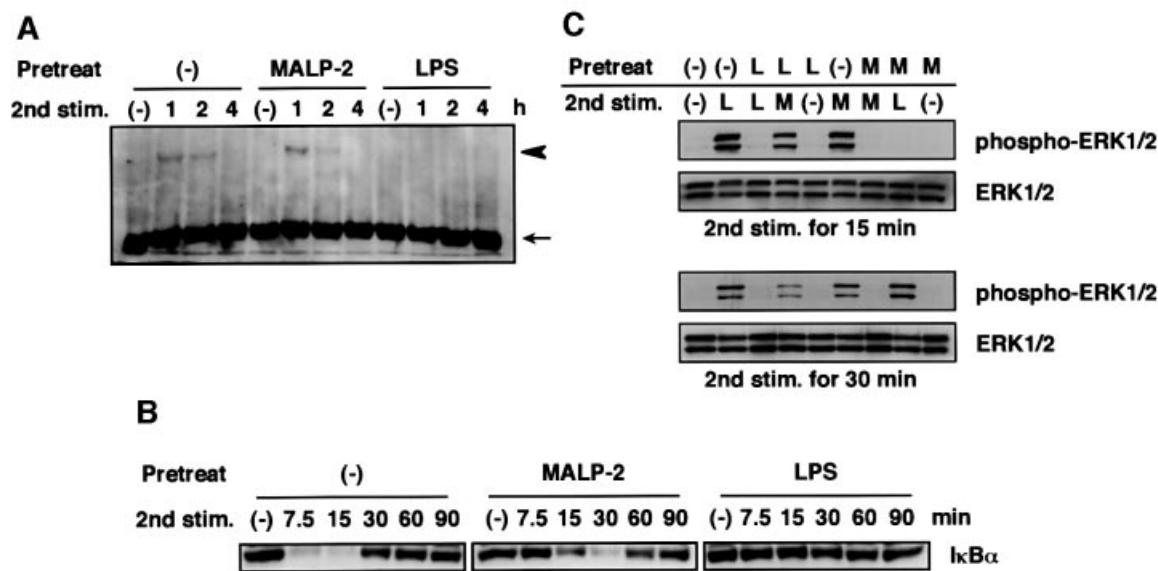


Fig. 2. Activation of IRF-3, NF-κB and ERK1/2 was observed in response to LPS in MALP-2-pretreated macrophages. (A) Peritoneal macrophages were pretreated with LPS or MALP-2 for 24 h. Cells were washed twice and then stimulated with LPS for the indicated periods. Cell lysates were prepared and subjected to native PAGE as described in Methods. Monomeric (arrow) and dimeric (arrowhead) forms of IRF-3 were detected by Western blotting. (B and C) Pretreated or untreated macrophages were re-stimulated with LPS for the indicated periods and then cell lysates were prepared. Western blotting was performed with anti-IκBα antibody (B) or anti-phospho-ERK1/2 antibody (C). The results are representative of three independent experiments that had similar results. L, LPS; M, MALP-2.

LPS, phosphorylation of ERK1/2 was not observed in either LPS- or MALP-2-pretreated cells (Fig.2C, upper). However, 30 min of re-stimulation with LPS induced the phosphorylation of ERK1/2 in MALP-2-, but not LPS-, pretreated cells (Fig. 2C, lower). Thus, a delayed activation of ERK1/2 in response to LPS was also observed in MALP-2-pretreated cells. In summary, the LPS-induced activation of IRF-3, NF-κB, and ERK1/2 was abolished in LPS-pretreated macrophages; however, the

activation of these molecules was observed in MALP-2-pretreated cells.

MALP-2 pretreatment resulted in impaired activation of IRAK1 in response to LPS

MALP-2-pretreated macrophages showed impaired LPS-induced expression of inflammatory mediators that are regu-

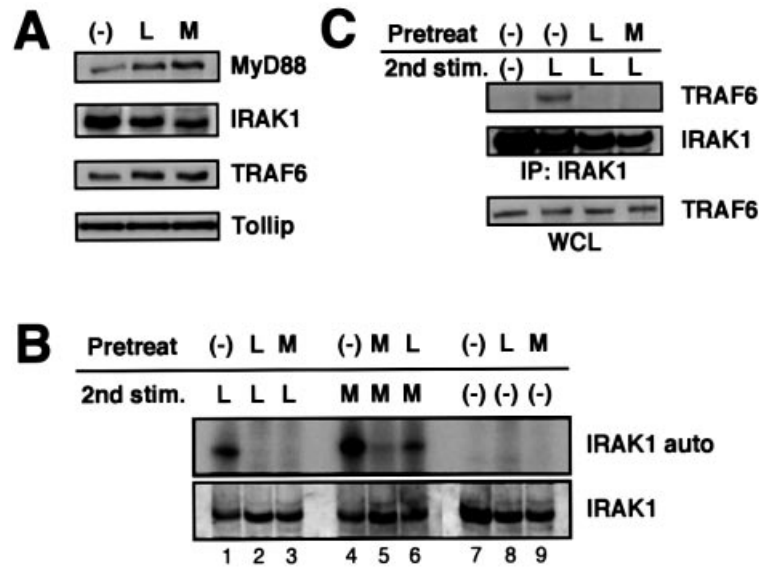


Fig. 3. LPS-induced IRAK1 activation was impaired in MALP-2-pretreated macrophages. (A) Peritoneal macrophages were cultured with LPS or MALP-2 for 24 h and then cell lysates were prepared. Lysate was separated by SDS-PAGE and subjected to Western blot analysis for the indicated molecules. (B) Cell lysates were immunoprecipitated with anti-IRAK1 antibody. IRAK1 activation was analyzed by *in vitro* kinase assay (upper panel). Expression of IRAK1 was analyzed by Western blot using anti-IRAK1 antibody (lower panel). (C) Cell lysates were immunoprecipitated with anti-IRAK1 antibody. Immunoprecipitants were separated by SDS-PAGE and subjected to Western blot analysis using anti-TRAF6 and anti-IRAK1 antibody to determine the association of IRAK1 and TRAF6 (top and middle panels). Expression of TRAF6 was determined by Western blot analysis (bottom panel). The results are representative of three independent experiments that had similar results. L, LPS; M, MALP-2.

lated by the MyD88-dependent signaling pathway. Therefore, we next analyzed whether the expression of components of the MyD88-dependent pathway such as MyD88, IRAK1, TRAF6 and Tollip was altered or not. Peritoneal macrophages were cultured in the presence or absence of LPS or MALP-2 for 24 h, and then the expression of MyD88, IRAK1, TRAF6 and Tollip was analyzed by Western blotting using specific antibodies. As shown in Fig. 3A, the expression of MyD88, TRAF6 and Tollip was not decreased, but rather slightly increased, in LPS- or MALP-2-treated cells. The expression of IRAK1 was, albeit slightly decreased, significantly observed in both of the pretreated cells. Next we assessed whether IRAK1 was activated in response to LPS or MALP-2 by *in vitro* kinase assay (Fig. 3B). When LPS-pretreated cells were stimulated with MALP-2, auto-phosphorylation of IRAK1 was clearly induced, although it was reduced when compared with that in non-pretreated cells (Fig. 3B, lanes 4 and 6). This is consistent with the finding that the stimulation of LPS-pretreated cells with MALP-2 induced the expression of genes for inflammatory cytokines (Fig. 1A, lane 4). However, when MALP-2-pretreated cells were stimulated with LPS, auto-phosphorylation of IRAK1 was not induced at all (Fig. 3B, lane 3).

In the IL-1 signaling pathway, IL-1 has been shown to induce the interaction of IRAK1 with TRAF6 (36). We analyzed the LPS-induced interaction of IRAK1 and TRAF6 in macrophages. Peritoneal macrophages were stimulated for 5 min, lysed and then immunoprecipitated with anti-IRAK1 antibody. The immunoprecipitant was blotted with anti-TRAF6 antibody (Fig. 3C). In non-pretreated cells, LPS stimulation induced the interaction between IRAK1 and TRAF6. In contrast, in both

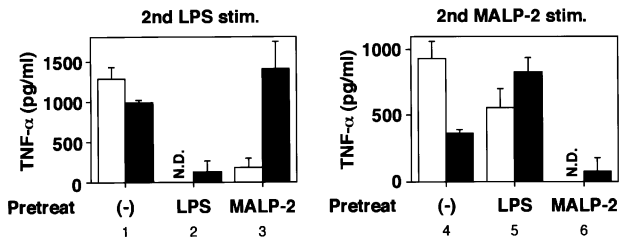


Fig. 4. MALP-2-induced LPS cross-tolerance was CHX sensitive. Peritoneal macrophages were pretreated with LPS or MALP-2 in the absence (open bar) or presence (closed bar) of 0.5 μg/ml of CHX for 24 h. Then cells were washed twice and stimulated with LPS or MALP-2 for an additional 24 h. After the second stimulation, the TNF-α concentration in the culture supernatants was measured by ELISA. The data are the average of three independent experiments. N.D., not detected.

LPS- and MALP-pretreated cells, no interaction was observed. Thus, MALP-2 pretreatment inhibits the activity of IRAK1 in response to LPS.

MALP-2-induced LPS tolerance was cycloheximide (CHX) sensitive

We next pretreated macrophages with MALP-2 or LPS in the presence or absence of CHX, an inhibitor of *de novo* protein synthesis, and analyzed the TNF-α production in response to a second stimulation (Fig. 4). LPS pretreatment in the presence of CHX significantly reduced the LPS-induced production of TNF-α (Fig. 4, lane 2). Thus, CHX did not have any influence on

the induction of LPS-induced LPS tolerance, which is consistent with our previous finding that LPS-induced LPS tolerance mainly occurs through down-regulation of the TLR4-MD2 complex. Similarly, MALP-2 pretreatment in the presence of CHX severely reduced TNF- α production in response to a second stimulation with MALP-2 (Fig. 4, lane 6). These results indicate that MALP-2-induced MALP-2 tolerance in macrophages may occur through down-regulation of TLR2, like the down-regulation of the TLR4-MD2 complex in LPS-induced LPS tolerance. On the other hand, when macrophages were pretreated with MALP-2 in the presence of CHX, the LPS-induced TNF- α production was the same as that in non-

pretreated cells (Fig. 4, lane 3). Thus, addition of CHX abolished the induction of MALP-2-induced LPS cross-tolerance, indicating that MALP-2 pretreatment induced LPS tolerance possibly by an unidentified MALP-2-induced factor(s) affecting the MyD88-dependent signaling pathways.

TLR7 ligand induced LPS tolerance with down-regulation of IRAK1 expression

MALP-2 (TLR2 ligand) was shown to induce LPS tolerance through a quite different mechanism from LPS-induced LPS tolerance. CpG DNA (TLR9 ligand) and IL-1, which utilize the common MyD88-dependent signaling pathway, have also been shown to induce LPS tolerance (12,17,18). From these findings, we speculate that various microbial components induce LPS tolerance. Recently, double-stranded RNA or poly(I:C) was shown to be recognized by TLR3 (37). Furthermore, TLR7 has been shown to recognize the imidazoquinoline compound, R-848, that has the potential to activate immune cells and produce inflammatory cytokines (33). Thus, R-848 can substitute for a microbial component recognized by TLR7. We pretreated macrophages with poly(I:C) or R-848 and analyzed the LPS-induced TNF- α production by ELISA (Fig. 5A). Poly(I:C) pretreatment did not reduce LPS-induced TNF- α production. In contrast, R-848 pretreatment resulted in severely reduced TNF- α production in response to LPS. We also analyzed LPS-induced TNF- α expression by Northern blotting (Fig. 5B). Pretreatment with R-848, but not poly(I:C), led to severely reduced expression of TNF- α mRNA in response to LPS.

We next examined the protein expression of MyD88, IRAK1, TRAF6 and Tollip in macrophages pretreated with R-848 or poly(I:C) (Fig. 6A). Poly(I:C) pretreatment did not induce any change in the expression of these proteins. In R-848-pretreated cells, the expression of MyD88, TRAF6 and Tollip was not markedly altered; however, the expression of IRAK1 was almost abolished. As a result, LPS-induced auto-phosphorylation of IRAK1 was not observed in R-848-pretreated cells, whereas LPS-induced IRAK1 activity was significantly increased in poly(I:C)-pretreated cells (Fig. 6B). Thus, R-848 pretreatment led to a severe reduction in IRAK1 expression and activity, which may cause the impaired LPS-induced TNF- α production.

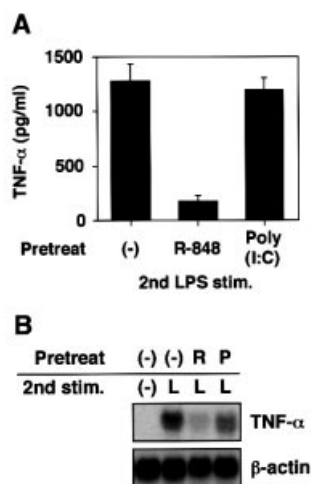


Fig. 5. Pretreatment with R-848, but not poly(I:C), led to the impaired LPS-induced TNF- α production. (A) Peritoneal macrophages were pretreated with 100 nM of R-848 or 100 μ g/ml of poly(I:C) for 24 h. Cells were washed twice and then stimulated with 100 ng/ml of LPS for 24 h. The TNF- α concentration in the culture supernatants was measured by ELISA. The data are the average of three independent experiments. (B) Peritoneal macrophages were pretreated with R-848 or poly(I:C) for 24 h. Cells were washed twice and then stimulated with LPS for 4 h. Total RNA was extracted and subjected to Northern blot analysis using mouse TNF- α cDNA probe. The same membrane was re-hybridized with mouse β -actin cDNA probe. The results are representative of three independent experiments that had similar results. L, LPS; R, R-848; P, poly(I:C).

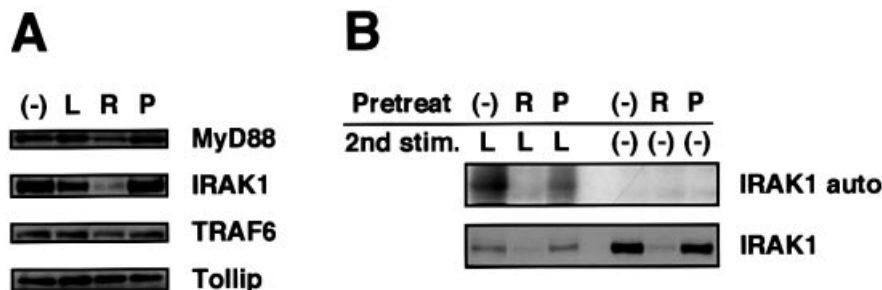


Fig. 6. Expression of IRAK1 was severely reduced in R-848-pretreated macrophages. (A) Macrophages were treated with the indicated stimuli for 24 h. Whole-cell lysates were prepared and subjected to Western blot analysis using antibodies for MyD88, IRAK1, TRAF6 and Tollip. (B) Cell lysates were immunoprecipitated with anti-IRAK1 antibody. The kinase activity of IRAK1 in the immunoprecipitates was measured by *in vitro* kinase assay (upper panel). The same lysates were blotted with anti-IRAK1 antibody to monitor the expression level of IRAK1 (lower panel). The results are representative of three independent experiments that had similar results. L, LPS; R, R-848; P, poly(I:C).

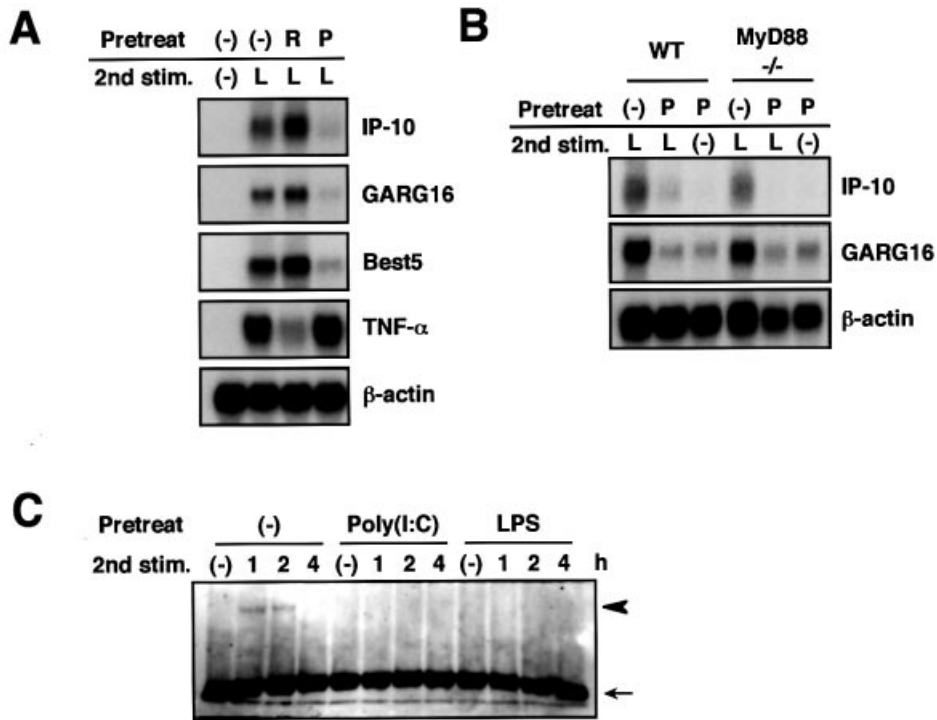


Fig. 7. Pretreatment with poly(I:C), but not R-848, led to the impaired LPS-induced expression of IFN-inducible genes. (A and B) Peritoneal macrophages were prepared from wild-type (A) or MyD88^{-/-} (B) mice, and then pretreated with the indicated stimuli for 24 h. Cells were washed twice and stimulated with LPS for 4 h. Total RNA was extracted and subjected to Northern blot analysis using cDNA probes for the indicated genes. (C) Peritoneal macrophages were pretreated with poly(I:C) or LPS for 24 h. Cells were washed twice and then stimulated with LPS for the indicated periods. Cell lysates were prepared and subjected to native PAGE. Monomeric (arrow) and dimeric (arrowhead) forms of IRF-3 were detected by Western blotting. The results are representative of three independent experiments that had similar results. L, LPS; R, R-848; P, poly(I:C).

Poly(I:C) pretreatment led to impaired LPS-induced expression of IFN-inducible genes

We next examined whether R-848 and poly(I:C) pretreatment affect the induction by LPS of IFN-inducible genes, such as IP-10, GARG16 and Best5. In contrast to the inhibition of LPS-induced TNF- α mRNA induction, R-848 pretreatment failed to block the expression of IFN-inducible genes in response to LPS, which is similar to the case for MALP-2 pretreatment (Fig. 7A). On the other hand, LPS-induced expression of IP-10, GARG16 and Best5 was not observed in poly(I:C)-pretreated cells. LPS-induced expression of the IFN-inducible genes was observed in MyD88^{-/-} mice (29). Therefore, peritoneal macrophages from MyD88^{-/-} mice were pretreated with poly(I:C) and analyzed for LPS-induction of the IFN-inducible genes (Fig. 7B). Poly(I:C) pretreatment led to impaired LPS-induced expression of the IFN-inducible genes in MyD88^{-/-} cells as well as wild-type cells. This indicates that the poly(I:C)-induced impairment of the LPS response occurs through a MyD88-independent manner.

We next analyzed the LPS-induced activation of IRF-3 in poly(I:C)-pretreated cells by native PAGE (Fig. 7C). In poly(I:C)-pretreated cells, the LPS-induced generation of IRF-3 homodimer was not observed at all. Thus, poly(I:C) pretreatment led to the impaired activation of IRF-3 in response to LPS.

Discussion

The LPS signaling pathway has now been being clarified. TLR4, possessing a TIR domain in the cytoplasmic portion, is essential for a receptor to trigger activation of the LPS-induced signaling pathway. An adaptor molecule, MyD88, which also harbors the TIR domain, associates with TLR4. Activation of TLR4 by LPS facilitates recruitment of IRAK1 and TRAF6 via MyD88. It leads to activation of JNK and NF- κ B, and thereby induces expression of the genes for inflammatory cytokines. This is clearly demonstrated in a study on MyD88^{-/-} mice, which did not show any inflammatory cytokine production in response to LPS (24). However, LPS-induced activation of JNK and NF- κ B was shown in MyD88^{-/-} mice, indicating the existence of MyD88-independent signaling (24). Recent studies have revealed that the LPS-induced activation of the MyD88-independent pathway leads to the expression of IFN-inducible genes and maturation of dendritic cells (28,29). Furthermore, another adaptor possessing the TIR domain has recently been identified, TIRAP/Mal (38,39). TIRAP/Mal has been shown to be involved in the LPS-induced maturation of dendritic cells, suggesting that it mediates MyD88-independent signaling (38).

In this study, we have precisely analyzed MyD88-dependent signaling (LPS-induced expression of genes for inflammatory cytokines) and MyD88-independent signaling (LPS-

induced expression of IFN-inducible genes) in macrophages pretreated with several microbial components. Consistent with our previous finding that LPS-induced LPS tolerance mainly occurs through down-regulation of the surface expression of the TLR4–MD2 complex, LPS pretreatment led to the impaired activation of both the MyD88-dependent and -independent signaling pathways (30). MALP-2 (TLR2 ligand) and R-848 (TLR7 ligand) affected the MyD88-dependent, but not the MyD88-independent, pathway. Poly(I:C) affected the MyD88-independent, but not the MyD88-dependent, pathway. The molecular mechanism for LPS tolerance has long been investigated; however, it remains unclear. This study clearly shows that LPS tolerance is induced by several different factors and mechanisms. This may account for the complicated nature of LPS tolerance.

LPS is a most potent activator of the immune system and exposure to an excess of LPS often induces multi-organ failure with a high mortality rate. In addition to our present findings, CpG DNA (TLR9 ligand) and IL-1, which utilize the same MyD88-dependent signaling pathway, induce a defective LPS response (12,17,18). Therefore, a variety of factors that activate TLR–MyD88 signaling induce changes in the LPS signaling pathway. The host may acquire mechanisms to effectively limit inflammatory responses by inducing tolerance to LPS through several TLR ligands. A recent publication described that LPS-induced tolerance was induced even in cells overexpressing TLR4 (40). We imagine that TLR4 and other TLR use a common signaling pathway via MyD88. Therefore, when TLR4 is constantly expressed, LPS-induced tolerance is achieved by a similar mechanism to TLR2- or TLR7-agonist-induced tolerance.

In THP-1 cell lines, LPS pretreatment has been shown to down-regulate the expression of IRAK1, a possible mechanism for LPS tolerance (32). In mouse peritoneal macrophages, LPS pretreatment slightly, but not significantly, reduced the expression of IRAK1. In contrast, R-848 (TLR7 ligand) pretreatment led to an almost complete loss of IRAK1 expression, which might be a major cause of LPS tolerance in R-848-pretreated cells. Thus, IRAK1 might be a major target for the induction of LPS tolerance. When we consider the fact that IRAK1 is ubiquitinated and degraded soon after either LPS or IL-1 β stimulation, ubiquitination of IRAK1 might account for the R-848-induced reduction in IRAK1 expression (32,41). At present, it remains unclear why only R-848 among several microbial components induced reduced the expression of IRAK1 in macrophages. TLR7-mediated signaling may have a unique system that is responsible for the reduced expression of IRAK1.

Another interesting finding is that poly(I:C) affected the MyD88-independent pathway. Poly(I:C) is representative of double-stranded RNA derived from RNA virus. It is intriguing why viral infection causes changes in the signaling pathway of LPS derived from Gram-negative bacteria. In this regard, we speculate as follows. Poly(I:C) induces the maturation of dendritic cells both in wild-type and in MyD88-deficient mice [(37) and our unpublished data]. Thus, poly(I:C) utilizes the MyD88-independent pathway. This pathway seems to be responsible for combating viral invasion, because it involves the activation of IRF-3 (29). IRF-3 was shown to be activated in response to viral infection and induce the expression of

several IFN-inducible genes (42–44). Therefore, viral infection may trigger activation of the MyD88-independent pathway for the host to cope with the virus. The virus, in turn, may affect the MyD88-independent pathway to evade the immune activation. It should be clarified why LPS, unlike other bacterial components, utilizes the MyD88-independent pathway in the future.

In this study, we have revealed that a variety of microbial components led to the impaired LPS-induced activation of the MyD88-dependent or -independent pathway through quite distinct mechanisms, which may account for the complicated nature of LPS tolerance.

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Abbreviations

CHX	cycloheximide
IRAK	IL-1 receptor-associated kinase
IRF	IFN-regulatory factor
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MALP-2	macrophage-activating lipopeptides-2 kDa
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor

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