Synergic Effects of Mycoplasmal Lipopeptides and Extracellular ATP on Activation of Macrophages

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Mycoplasmal lipopeptides *S*-(2,3-bispalmitoyloxypropyl)-CGDPKHSPKSF and *S*-(2,3-bispalmitoyloxypropyl)-CGNNDESNISFKEK activated a monocytic cell line, THP-1 cells, to produce tumor necrosis factor alpha. The activity of the lipopeptides was augmented by ATP in a dose-dependent manner. In addition, the level of expression of mRNAs for tumor necrosis factor alpha and interleukin-1β, -6, and -8 was also upregulated by the lipopeptides and/or extracellular ATP, but that of interleukin-10 was not. The P2X purinergic receptor antagonists pyridoxal phosphate 6-azophenyl 2′,4′-disulfonic acid and periodate-oxidized ATP suppressed the activity of ATP to augment the activation of THP-1 cells by the lipopeptides, suggesting that P2X receptors play important roles in the activity of ATP. The nuclear factor κB inhibitor dexamethasone also suppressed the activity, suggesting that the activity of ATP is dependent upon the nuclear factor κB. Thus, these results suggest that the interaction of extracellular ATP with the P2X receptors is attributed to the activity of ATP to augment the activation of THP-1 cells by mycoplasmal lipopeptides.

Mycoplasmas are cell wall-less and the smallest self-replicating microorganisms. Although they do not possess bacterial modulins such as lipopolysaccharides (LPSs), lipoteichoic acids, or peptidoglycans, they are capable of activating macrophages/monocytes and fibroblasts (11, 39). Recently, the membrane-bound lipoproteins of mycoplasmas were found to play important roles in activation of monocytes/macrophages and fibroblasts by mycoplasmas (11, 18, 24, 30, 31, 40). Furthermore, the active site of mycoplasmal lipoproteins was identified as the N-terminal lipopeptide moieties (31, 40).

S-(2,3-Bispalmitoyloxypropyl)-CGNNDESNISFKEK (MALP-2) purified from *Mycoplasma fermentans* was first characterized as a macrophage-activating lipopeptide (31). We have also characterized *S*-(2,3-bispalmitoyloxypropyl)-CGDPKHSPKSF, called FSL-1, synthesized on the basis of the structure of a 44-kDa lipoprotein of *Mycoplasma salivarium* responsible for activation of human gingival fibroblasts (40). FSL-1 can activate monocytes/macrophages as well as human gingival fibroblasts (40). Thus, mycoplasmal lipopeptides possess several biological activities which are shared by LPS, although the activities of the lipopeptides are not inhibited by polymyxin B, which abrogates the activities of LPS (11).

Recently we found that signaling by FSL-1 was mediated by Toll-like receptor 2 (TLR2) (unpublished data), in accordance with previous data that TLR2 is a receptor for bacterial lipoproteins and MALP-2 (7, 17, 23, 44, 45). Members of the TLR family recently emerged as candidate receptors capable of transmitting signaling by pathogen-associated molecular

patterns such as LPS, lipoteichoic acid, peptidoglycans, bacterial CpG DNA, and lipoproteins (2, 27). TLR4 has been well characterized as a receptor for LPS (5, 8, 46, 49). These findings also suggest that mycoplasmal lipopeptides activate mammalian cells by a mechanism different from that of LPS.

Several studies have also suggested that activation of macrophages by LPS is regulated by extracellular ATP and purinergic receptors, P2X$_7$, and periodate-oxidized ATP (oATP) (4, 14, 19, 20, 28, 37). In addition, ATP-gated ionotropic (P2X) receptors, including the P2X$_7$ receptor, were confirmed to be expressed on the cell surface of macrophages (21, 22, 35).

In this study, therefore, experiments were carried out to examine the effects of extracellular ATP on activation of monocytes/macrophages by mycoplasmal lipopeptides.

MATERIALS AND METHODS

Reagents. ATP was obtained from Molecular Probes (Eugene, Oreg.). Pyridoxal phosphate 6-azophenyl 2′,4′-disulfonic acid (PPADS), oATP, and dexamethasone (DEX) were purchased from Sigma-Aldrich (St. Louis, Mo.). All other chemicals were obtained from commercial sources and were of analytical or reagent grade.

Synthesis of FSL-1 and MALP-2. FSL-1 and MALP-2 were synthesized as follows. The side chain-protected peptide GDPKHPKSF or GNNDESNISFKEK was built up with an automated peptide synthesizer, model 433 (Applied Biosystems, Foster City, Calif.). 9-Fluorenylmethoxy carbonyl (Fmoc)-*S*-(2,3-bispalmitoyloxypropyl)-cysteine (Novabiochem, Laeufelfingen, Switzerland) was manually coupled to the peptide resin using a solvent system of 1-hydroxy-7-azabenzotriazole-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/CH$_2$Cl$_2$-DMF. The Fmoc and resin were removed from the lipopeptide by trifluoroacetic acid. The lipopeptides were purified by preparative high-pressure liquid chromatography (HPLC) with a reverse-phase C$_{18}$ column (30 by 250 mm). The purity of FSL-1 and MALP-2 was confirmed by analytical HPLC with a reverse-phase C$_{18}$ column (4.6 by 150 mm) to be 97 and 98%, respectively.

Determination of TNF-α in the culture supernatant. A human acute monocytic leukemia cell line, THP-1 (47), was purchased from Health Science Research Resources Bank (Osaka, Japan). Cells were grown at 37°C in a humidified atmosphere of 5% CO$_2$ in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). A 1-ml volume of cell suspension (10^6 cells/ml) of THP-1 cells was seeded in each well

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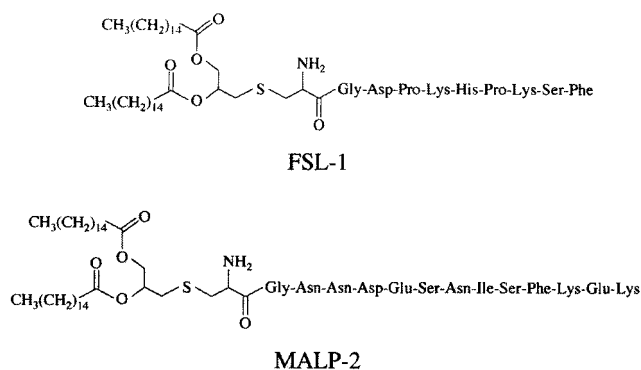
FIG. 1. Structures of FSL-1 and MALP-2.

94°C for 30 s, amplifications were carried out for 28 cycles as follows: denaturation at 94°C for 30 s, annealing at primer-specific temperatures (see below) for 30 s, and extension at 72°C for 90 s. After the final PCR cycle, extension was allowed to proceed at 72°C for 2 min. The annealing temperatures were 54°C for $P2X_7$, 55°C for β-actin, TNF-α, IL-1β, IL-6, IL-8, IL-10, $P2Y_{11}$, TLR2, and TLR6, 64°C for $P2X_1$, and 67°C for $P2Y_2$.

The PCR products were electrophoresed on a 2% gel of NuSieve 3:1 agarose in 0.5× Tris-borate-EDTA buffer containing 5 μg of ethidium bromide per ml. The stained PCR products were photographed under UV light. The net intensity of gene-specific PCR products was analyzed by using a Kodak IS 440 CF image analyzer (Kodak, Rochester, N.Y.).

Determination of ATP in the culture supernatant. A 1-ml volume of cell suspensions (10^6 cells/ml) of THP-1 cells was seeded in each well of a 24-well tissue culture plate. Cells were stimulated at 37°C for 8 h with various concentrations of mycoplasmal lipopeptides. The amount of ATP in the culture supernatant was measured with an ATP determination kit (Molecular Probes) according to the manufacturer's instructions.

of a 24-well tissue culture plate. The cells were stimulated at 37°C for 1 h with 10 nM FSL-1 or 50 nM MALP-2 or unstimulated and incubated at 37°C for 8 h after the addition of various concentrations of ATP. The amount of tumor necrosis factor alpha (TNF-α) in the culture supernatant was measured by an enzyme-linked immunosorbent assay (ELISA) using a human TNF-α Cytoset (Biosource, Camarillo, Calif.) according to the manufacturer's instructions.

Expression of mRNAs of cytokines and purinergic receptors in THP-1 cells by reverse transcription-coupled PCR. Oligonucleotide primers for interleukin-1β (IL-1β), IL-6, IL-8, IL-10, TNF-α, and β-actin for PCR amplification were synthesized according to the sequences described previously (25). Primers specific for TLR2, TLR6, and the purinergic P2 receptors $P2X_1$, $P2X_7$, $P2Y_2$, and $P2Y_{11}$ were synthesized on the basis of the sequences described by Zhang et al. (49) and Adrian et al. (1), respectively. The specificity of these primers was confirmed by Southern hybridization with a probe coding for internal sequence.

THP-1 cells (3×10^6) in 3-ml volumes of RPMI 1640 medium were stimulated at 37°C for 1 h with 10 nM FSL-1 or unstimulated and then incubated at 37°C for various periods of time after the addition of 500 μM ATP. Total RNA from THP-1 cells was prepared by using an RNeasy kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer's instructions. The quantity of mRNA was determined photometrically at 260 nm. RNA (approximately 0.8 μg) was reverse transcribed to cDNA in a 20-μl reaction volume containing a 1 μM concentration of each of the antisense primers using an RNA PCR kit (AMV version 2.1; Takara Biochemicals, Shiga, Japan) according to the manufacturer's instructions.

The PCRs were performed in 50-μl final volumes containing 10 μl of cDNA, 2.5 mM MgCl$_2$, and 20 pmol of each sense primer. After initial denaturation at

RESULTS

Augmentation of mycoplasmal lipopeptide-induced activation of THP-1 cells by ATP added extracellularly. The synthetic mycoplasmal lipopeptides FSL-1 and MALP-2 (Fig. 1) are potent activators of monocytes/macrophages (18, 24, 31, 40). The differences in the structures of FSL-1 and MALP-2 are the amino acid sequence and length of the peptide portion (Fig. 1). An experiment was carried out to determine whether ATP added extracellularly promotes FSL-1- or MALP-2-induced activation of THP-1 cells, because activation of macrophages by LPS is upregulated by ATP (4, 14, 19, 20, 28, 37). The activation of THP-1 cells by FSL-1 or MALP-2 assessed by TNF-α production was augmented by ATP in a dose-dependent manner (Fig. 2). The TNF-α production-inducing activity of FSL-1 and MALP-2 was enhanced approximately twofold by 500 μM ATP. In the absence of lipopeptides, ATP activated THP-1 cells (Fig. 2). In addition, RT-PCR analysis demonstrated that the expression level of IL-1β, IL-6, and IL-8 as well as TNF-α mRNAs was also enhanced by ATP, whereas that of IL-10 was not (Fig. 3).
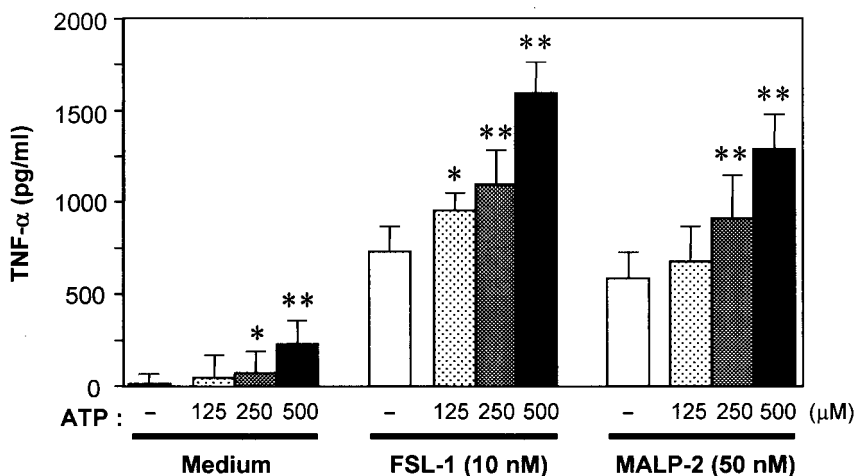

FIG. 2. Effects of extracellular ATP on the activity of mycoplasmal lipopeptides to induce TNF-α production by THP-1 cells. THP-1 cells (10^6 cells/ml) were treated at 37°C for 1 h with 10 nM FSL-1 or 50 nM MALP-2 or not treated, and then ATP was added to the culture at the indicated concentrations. After an 8-h incubation period, the culture supernatant was collected and assayed for the amount of TNF-α by ELISA. Results, expressed as the means ± standard deviations (SD) of values for triplicate wells, are representative of three separate experiments. Statistical significance of augmentation by ATP, compared with the ATP-nontreated cells (open bars), was analyzed by t test. *, $P < 0.05$; **, $P < 0.01$.
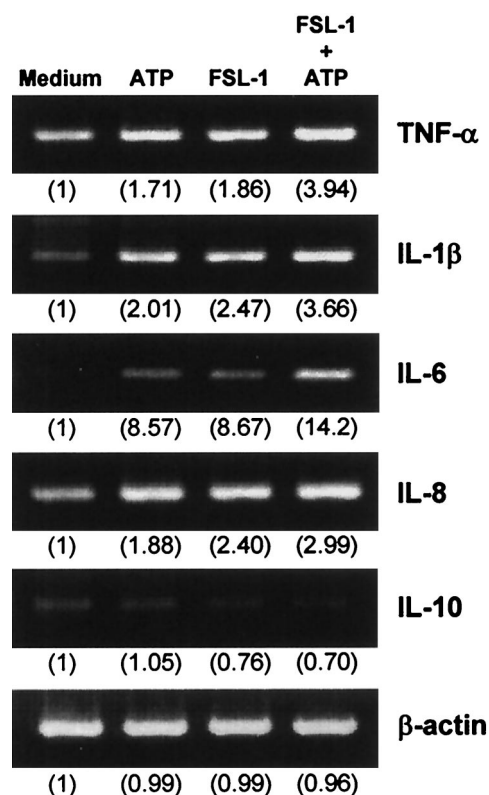
FIG. 3. Transcription of cytokine genes in THP-1 cells stimulated with FSL-1 and/or ATP or not. THP-1 cells (3×10^6) were stimulated at 37°C for 1 h with 10 nM FSL-1 or not and incubated for 1 h after the addition of 500 μM ATP. The expression of TNF-α, IL-1β, IL-6, IL-8, and IL-10 mRNAs was analyzed by RT-PCR. The identity of the PCR products was confirmed by Southern hybridization with a probe coding for the internal sequence (data not shown). Values in parentheses indicate the intensities of the signals of the mRNAs obtained by densitometric analysis, with the intensity of the signal of the mRNAs of β-actin or various cytokines in the cells in the absence of the stimulators (medium) being taken as 1.

FIG. 4. Transcription of ATP-sensitive purinergic receptor genes in THP-1 cells stimulated with FSL-1 and/or ATP or not. THP-1 cells (3×10^6) were stimulated at 37°C for 1 h with 10 nM FSL-1 or not and incubated for 8 h after the addition of 500 μM ATP. The expression of mRNAs of the ATP-sensitive P2 receptors $P2X_1$, $P2X_7$, $P2Y_2$, and $P2Y_{11}$ was analyzed by RT-PCR. The identity of the PCR products was confirmed by Southern hybridization with a probe coding for the internal sequence (data not shown). Values in parentheses indicate intensities of the signals of the mRNAs obtained by densitometric analysis, taking the intensity of the signal of the mRNAs of various P2 receptors in the cells in the absence of the stimulators (medium) as 1.

Thus, these results suggest that ATP augments mycoplasmal lipopeptide-induced activation of macrophages by its interaction with some ATP receptors.

mRNA expression of ATP-sensitive P2 purinergic receptors on THP-1 cells. Extracellular nucleotides bind to cell surface receptors, which are designated purinergic P2 receptors (12, 16). Several P2 receptors are divided into two groups: P2X receptors, ligand-gated cation channels, and P2Y receptors coupled to G proteins (16, 35, 48). It was reported that P2X and P2Y receptor subtypes, including $P2X_1$, $P2X_7$, $P2Y_2$, and $P2Y_{11}$, which are sensitive to ATP (26, 35, 42, 48), regulate differentiation, activation, or proliferation of immunocytes (1, 3, 10, 12, 35, 48). Therefore, the expression of these ATP-sensitive P2 receptor mRNAs was examined by RT-PCR in THP-1 cells stimulated with FSL-1 and/or ATP or unstimulated. $P2X_1$, $P2X_7$, $P2Y_2$, and $P2Y_{11}$ genes were transcribed in THP-1 cells irrespective of stimulation (Fig. 4).

Therefore, the effects of PPADS and oATP on the enhancement of the TNF-α production-inducing activity of the lipopeptides by extracellular ATP were examined, because PPADS is an antagonist for P2X receptors ($P2X_1$ to $P2X_7$) and
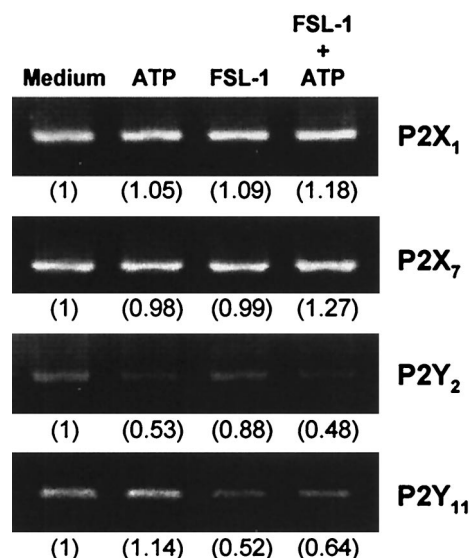
some P2Y receptors except for $P2Y_2$ and $P2Y_{11}$ (26, 29) and oATP is an antagonist for P2X receptors, especially $P2X_7$ (4, 14, 19, 28, 29, 32). Both PPADS and oATP suppressed the enhancement by ATP in a dose-dependent manner (Fig. 5A). Furthermore, oATP suppressed the activity more strongly than PPADS. The activity obtained by oATP treatment was significantly lower than that of FSL-1 in the absence of extracellular ATP (Fig. 5A). Therefore, the effects of oATP on the TNF-α production-inducing activity of FSL-1 were examined. As a result, it was found that oATP inhibited the activity of FSL-1 even in the absence of extracellular ATP (Fig. 5B).

These results suggest that P2X receptors, especially a $P2X_7$ receptor, are involved in the modulation of FSL-1-induced activation of macrophages by extracellular ATP.

Effects of extracellular ATP and FSL-1 on the mRNA expression of TLR2 or TLR6 in THP-1 cells. It was reported that signaling by MALP-2 is mediated by TLR2 (2, 44). We have also found that a receptor for FSL-1 is TLR2 (unpublished data). Recently, mycoplasmal lipopeptides were shown to be discriminated by TLR6 (45). Therefore, the effects of extracellular ATP or FSL-1 on the expression of TLR2 and -6 were examined. It was confirmed that TLR2 and TLR6 mRNAs were expressed in the cells, but the expression level was not enhanced by stimulation with ATP and/or FSL-1 (Fig. 6).

Effects of DEX on TNF-α production-inducing activity of FSL-1 and/or ATP. TLRs activate kinase cascades which result in nuclear translocation of NF-κB (2, 5, 27, 41, 49). It was demonstrated that extracellular ATP is capable of inducing activation of the NF-κB subunit p65 (RelA) via the $P2X_7$
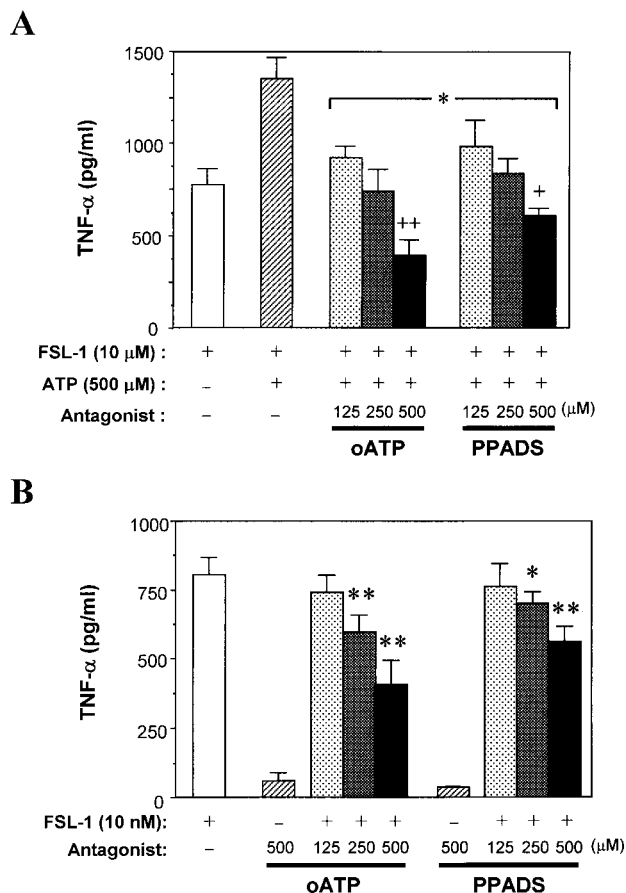
A

B

FIG. 5. Effects oATP and PPADS on TNF-α production by THP-1 cells stimulated with FSL-1 and/or ATP. (A) THP-1 cells (10^6cells/ml) were treated at 37°C for 1 h with oATP or PPADS at 125, 250, and 500 μM and then incubated for 8 h after the addition of 10 nM FSL-1 and 500 μM ATP. The culture supernatant was collected and assayed for the amount of TNF-α by ELISA. Results, expressed as the means ± SD of triplicate wells, are representative of three separate experiments. Statistical significance was analyzed by t test. *, $P < 0.01$ versus hatched bar; +, $P < 0.05$ versus open bar; ++, $P < 0.01$ versus open bar. (B) THP-1 cells (10^6 cells/ml) were treated at 37°C for 1 h with 125, 250, and 500 μM oATP or PPADS and then incubated for 8 h after the addition of 10 nM FSL-1. The culture supernatant was collected and assayed for the amount of TNF-α by ELISA. Results, expressed as the means ± SD of triplicate wells, are representative of three separate experiments. Statistical significance was analyzed by t test. *, $P < 0.05$ versus open bar; **, $P < 0.01$ versus open bar.

FIG. 6. Effects of FSL-1 and/or ATP on the transcription of TLR2 or TLR6 mRNA in THP-1 cells. THP-1 cells (3×10^6) were stimulated at 37°C for 1 h with 10 nM FSL-1 or not and incubated for 8 h after the addition of 500 μM ATP. The expression of TLR2 mRNA was analyzed by RT-PCR. The identity of the PCR products was confirmed by Southern hybridization with a probe coding for the internal sequence (data not shown). Values in parentheses indicate intensities of the signals of the mRNAs obtained by densitometric analysis, taking the intensity of the signal of the mRNA of TLR2 or TLR6 in the cells in the absence of the stimulators (medium) as 1.

release of ATP from THP-1 cells. THP-1 cells constitutively released ATP, producing extracellular concentrations of approximately 4 nM when assayed at 10^6 cells in 1 ml. However, THP-1 cells did not release additional ATP during an 8-h stimulation period with FSL-1 or MALP-2 (Fig. 8).

DISCUSSION

This is the first study to report the effects of extracellular ATP on mycoplasmal lipopeptide-induced activation of macrophages. FSL-1 is capable of inducing production of TNF-α

receptor (15). Therefore, the effects of the NF-κB inhibitor DEX on the TNF-α production-inducing activity of FSL-1 and/or ATP on THP-1 cells were examined. DEX markedly inhibited the activity of FSL-1 and/or ATP (Fig. 7). The same result was also obtained when MALP-2 was used as a stimulator (data not shown). This result suggests that TNF-α production by THP-1 cells induced by mycoplasmal lipopeptides or ATP is mediated by NF-κB activation.

Effects of lipopeptides on extracellular ATP concentrations. The findings obtained in this study suggest that extracellular ATP plays important roles in the enhancement of FSL-1-induced activation of THP-1 cells. Therefore, an experiment was carried out to test whether lipopeptide stimulation causes the
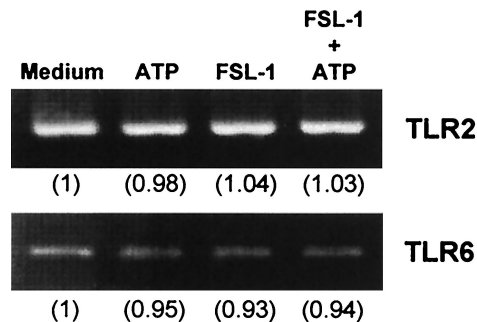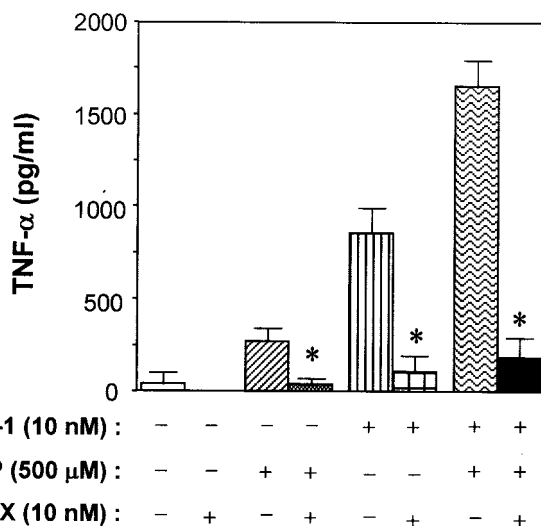

FIG. 7. Effects of DEX on TNF-α production by THP-1 cells induced by FSL-1 and/or ATP. THP-1 cells (10^6 cells/ml) were treated at 37°C for 1 h with 10 nM DEX and then incubated for 8 h after the addition of 10 nM FSL-1 and/or 500 μM ATP. The culture supernatant was collected and assayed for the amount of TNF-α by ELISA. Results, expressed as the means ± SD of triplicate wells, are representative of three separate experiments. Statistical significance was analyzed by t test. *, $P < 0.005$ versus the absence of DEX.
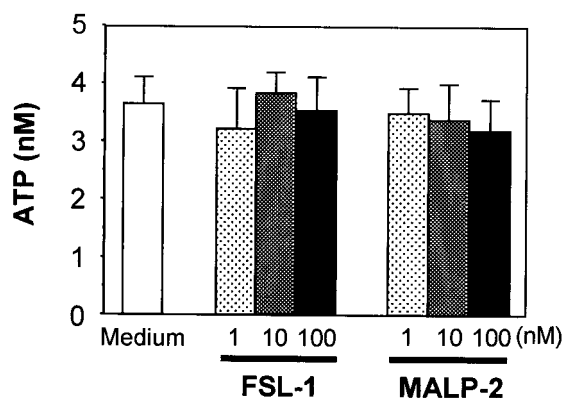
FIG. 8. Determination of ATP in the culture of THP-1 cells stimulated with lipopeptides or not. THP-1 cells (10^6 cells/ml) were treated at 37°C for 8 h with 1 to 100 nM FSL-1 or MALP-2. The culture supernatant was collected and assayed for the amount of ATP. Results, expressed as the means ± SD of triplicate wells, are representative of three separate experiments.

and IL-6 by THP-1 cells and ICAM-1 expression on the cell surface of human gingival fibroblasts (40). MALP-2 is capable of activating macrophages to release nitric oxide and produce TNF-α, IL-1, and IL-6 (18, 24, 31). It was demonstrated that signaling by MALP-2 and FSL-1 is mediated by TLR2. More recently, Takeuchi et al. demonstrated that macrophages prepared from TLR6 knockout (TLR6$^{-/-}$) as well as TLR2$^{-/-}$ mice are unresponsive to MALP-2, suggesting that mycoplasmal lipopeptide is recognized by dual receptors consisting of TLR2 and TLR6 (45).

A similar result was obtained when FSL-1 was used as a stimulator of macrophages prepared from these knockout mice (unpublished data). Furthermore, the expression of TLR2 and TLR6 mRNAs was confirmed in THP-1 cells (Fig. 6). Taken together, our findings that mycoplasmal lipopeptide-induced activation of macrophages is augmented by the addition of ATP suggest that there is cross talk between the signaling pathways triggered by TLR2 and -6 and ATP receptors, especially P2X$_7$, which leads to TNF-α production. Indeed, signaling transmitted by both TLRs and P2X$_7$ leads to translocation of NF-κB, which regulates production of TNF-α (2, 5, 15, 27, 41, 49).

It was previously demonstrated that TLR signaling involves steps that are similar to those used by the IL-1 receptor (2, 27, 49). These common steps include the involvement of the adaptor molecule MyD88 and the serine kinase IL-1R-associated kinase, which interacts with an adaptor known as TRAF6 (43, 49). TRAF6 links to the mitogen-activated protein (MAP) kinase kinase kinase TAK1 through an adaptor, TAB (34, 41). TAK1 is involved in activation of the transcription factor NF-κB through the activation IκB kinase and in the activation of AP-1 transcription family members Jun and Fos via additional MAP kinases (34, 41, 43, 49). We also confirmed that mycoplasmal lipopeptides FSL-1 and MALP-2 also activate NF-κB and AP-1 (33). Furthermore, it was reported that extracellular ATP also activates NF-κB through the P2X$_7$ receptor by selectively targeting NF-κB subunit p65 (RelA) (15).

In the present study, we demonstrated that DEX treatment significantly attenuated the secretion of TNF-α by macro-

phages induced by mycoplasmal lipopeptides and/or ATP (Fig. 7). DEX is one of the synthetic glucocorticoids that selectively inhibit NF-κB/Rel and AP-1 (13, 38). Therefore, it is suggested that the inhibitory activity of DEX is attributed to inhibition of NF-κB activation induced by signaling through TLR2/6 and/or the ATP receptor, which results in the repression of TNF-α production by macrophages.

The TNF-α production-inducing activity of FSL-1 obtained by oATP treatment was significantly lower than that of FSL-1 in the presence of extracellular ATP (Fig. 5A). Therefore, the effects of oATP on the activity of FSL-1 were examined, and it was found that oATP inhibited the activity of FSL-1 even in the absence of extracellular ATP (Fig. 5B). These results suggest two possibilities: one is that a small amount of ATP is included in the culture medium and the ATP enhanced the activity of FSL-1; and the other is that FSL-1 is able to interact with the receptors such as P2X$_7$ to which oATP binds. However, the former possibility seems less possible, because approximately 4 nM ATP in the culture medium (Fig. 8) is too weak to induce the same amount of TNF-α as that suppressed by oATP, judging from the result shown in Fig. 2.

Recently it has been demonstrated that the C-terminal part of the P2X$_7$ receptor forms a domain that has the potential to bind LPS in a manner similar to that observed with the LPS-binding domain of LPS-binding protein, and indeed, LPS can bind the peptide synthesized on the basis of the amino acid sequence of the C-terminal part of P2X$_7$ in vitro (9). Therefore, the latter possibility may be more likely, that FSL-1 interacts with P2X$_7$ receptor, because both LPS and FSL-1 have common lipid moieties responsible for the expression of their biological activities.

It was found that the macrophage response to LPS is modulated by extracellular ATP (4, 14, 19, 20, 28, 37). Beigi and Dubyak reported that activation of macrophages by LPS does not induce ATP release and autocrine stimulation of P2 receptors (4). The present study also showed that extracellular ATP enhanced mycoplasmal lipopeptide-induced activation of macrophages, and the macrophages did not induce additional ATP release during stimulation with these lipopeptides (Fig. 8). Thus, mycoplasmal lipopeptides function as an activator of macrophages without induction of ATP release in the same way as LPS. Many studies have demonstrated that under acute inflammatory condition or mechanical stress, various types of cells induce release of ATP (6, 36), although the mechanism remains unknown. Therefore, it is very likely that an inflammatory response induced by bacterial products such as LPS or lipopeptide is enhanced in vivo by ATP released in the lesions.

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