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Shintaro Sato, Fumiko Nomura, Taro Kawai, Osamu  
Takeuchi, Peter F. Mùhlradt, Kiyoshi Takeda and Shizuo  
Akira

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# Synergy and Cross-Tolerance Between Toll-Like Receptor (TLR) 2- and TLR4-Mediated Signaling Pathways<sup>1</sup>

Shintaro Sato,\*<sup>‡</sup> Fumiko Nomura,\*<sup>‡</sup> Taro Kawai,\*<sup>‡</sup> Osamu Takeuchi,\*<sup>‡</sup> Peter F. Muhlradt,<sup>†</sup> Kiyoshi Takeda,\*<sup>‡</sup> and Shizuo Akira<sup>2\*‡</sup>

A family of Toll-like receptor (TLR) mediates the cellular response to bacterial cell wall components; murine TLR2 and TLR4 recognize mycoplasmal lipopeptides (macrophage-activating lipopeptides, 2 kDa (MALP-2)) and LPS, respectively. Costimulation of mouse peritoneal macrophages with MALP-2 and LPS results in a marked increase in TNF- $\alpha$  production, showing the synergy between TLR2- and TLR4-mediated signaling pathways. Macrophages pretreated with LPS show hyporesponsiveness to the second LPS stimulation, termed LPS tolerance. The LPS tolerance has recently been shown to be primarily due to the down-regulation of surface expression of the TLR4-MD2 complex. When macrophages were treated with MALP-2, the cells showed hyporesponsiveness to the second MALP-2 stimulation, like LPS tolerance. Furthermore, macrophages pretreated with MALP-2 showed reduced production of TNF- $\alpha$  in response to LPS. LPS-induced activation of both NF- $\kappa$ B and c-Jun NH<sub>2</sub>-terminal kinase was severely impaired in MALP-2-pretreated cells. However, MALP-2-pretreated macrophages did not show any reduction in surface expression of the TLR4-MD2 complex. These findings indicate that LPS-induced LPS tolerance mainly occurs through the down-regulation of surface expression of the TLR4-MD2 complex; in contrast, MALP-2-induced LPS tolerance is due to modulation of the downstream cytoplasmic signaling pathways. *The Journal of Immunology*, 2000, 165: 7096–7101.

**B**acterial cell wall components activate monocytes and macrophages to produce several inflammatory cytokines such as TNF- $\alpha$  and IL-6. Among the bacterial cell wall components, LPS from the cell wall of Gram-negative bacteria is best known as a component that activates monocytes and macrophages. Excessive activation of monocytes and macrophages by LPS leads to endotoxin shock, a systemic serious disorder with a high mortality rate in human and experimental animals. Pre-exposure to LPS reduces sensitivity to a second challenge with LPS, called LPS tolerance (also called LPS hyporesponsiveness, or refractoriness) (1). Animals pretreated with low doses of LPS showed reduced febrile response and mortality rate after a second challenge with LPS. LPS tolerance was also observed at the level of macrophages. Macrophages pretreated with LPS showed no or reduced production of inflammatory cytokines in response to the second stimulation with LPS. Although molecular mechanisms of LPS tolerance have long been investigated, they remain unclear.

Several bacterial cell wall components as well as LPS have been shown to possess a potential to activate monocyte and macrophages and induce a symptom like endotoxin shock in experimental animals. These include bacterial lipopeptides, peptidoglycan, muramyl dipeptide from Gram-positive bacteria, and bacterial DNA containing unmethylated CpG motif. These bacterial com-

ponents have also been shown to induce tolerance to the subsequent stimulation (2–4).

Recent studies have demonstrated that bacterial cell wall components are recognized by pattern recognition receptors on innate immune cells (5, 6). Especially, families of Toll-like receptors (TLR)<sup>3</sup> have been shown involved in the recognition of bacterial components. In *Drosophila*, Toll family proteins specify the innate immune responses to microbial infections; Toll is responsible for the response to fungal infection, whereas 18-wheeler responds to bacterial infection (7, 8). Mutations in the *Tlr4* gene have been found in LPS-hyporesponsive C3H/HeJ and C57BL/10ScCr mice (9, 10). Furthermore, analyses of gene-targeted mice have recently demonstrated that the TLR family recognizes the specific pattern of bacterial cell wall components (11–13). Recent studies reported that modulation of the signaling pathway via TLR4 is involved in development of LPS tolerance (14, 15). We have also analyzed the molecular mechanisms of LPS tolerance (16). When mouse peritoneal macrophages were treated with LPS, surface expression of the TLR4-MD2 complex was severely reduced, which led to hyporesponsiveness to LPS.

In the present study, we investigated the synergy and cross-tolerance between LPS and mycoplasmal lipopeptides (macrophage-activating lipopeptides, 2 kDa (MALP-2)), each of which is differentially recognized by TLR4 and TLR2, respectively.

## Materials and Methods

### Cells

Peritoneal macrophages were isolated from C57BL/6J mice, ICR mice (SLC, Shizuoka, Japan), or IL-10-deficient mice (The Jackson Laboratory, Bar Harbor, ME). Briefly, mice were i.p. injected with 2 ml of 4% thioglycolate. After 3 days of injection, 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.

\*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; <sup>†</sup>Immunobiology Research Group, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany; and <sup>‡</sup>Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan  
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<sup>2</sup> Address correspondence and reprint requests to Dr. Shizuo Akira, Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail address: sakira@biken.osakau.ac.jp

<sup>3</sup> Abbreviations used in this paper: TLR, Toll-like receptor; MALP-2, macrophage-activating lipopeptides, 2 kDa; ERK, extracellular-regulated kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; iNOS, inducible NO synthase; IRAK, IL-1 receptor-associated kinase.

RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Life Technologies), 2-ME (100  $\mu$ M), penicillin (5 U/ml), and streptomycin (50 ng/ml) was used as culture medium.

### Reagents and Abs

LPS from *Salmonella minnesota* Re595 prepared by a phenol-chloroform-petroleum ether extraction procedure was purchased from Sigma (St. Louis, MO). LPS from *Escherichia coli* serotype O55:B5 was obtained from List Biological Laboratories (Campbell, CA). MALP-2 was synthesized and purified as described previously (13).

The MTS510 mAb that specifically recognizes the mouse TLR4-MD2 complex was provided by K. Miyake (17). Anti-IL-1-receptor-associated kinase (IRAK) 1 Ab was provided by Hayashibara Biochemical Laboratories (Okayama, Japan). Rabbit anti-c-Jun NH<sub>2</sub>-terminal kinase 1 (anti-JNK), anti-extracellular-regulated kinase 1 and 2 (anti-ERK-1,2), anti-p50, and anti-p65 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-ERK-1,2 Ab was obtained from New England Biolabs (Beverly, MA).

### Measurement of cytokine concentration

Peritoneal macrophages ( $1 \times 10^5$ ) were stimulated with the indicated concentrations of LPS and/or MALP-2 for 24 h. For tolerance experiment, cells were preincubated with 100 ng/ml LPS or 3 ng/ml MALP-2 for 24 h and washed twice with culture medium. Then cells were stimulated with 100 ng/ml LPS or 3 ng/ml MALP-2 for 24 h. Concentrations of TNF- $\alpha$  in the culture supernatants were measured by ELISA according to the manufacturer's instructions (Genzyme Techno, Minneapolis, MN).

### Northern blot analysis

Peritoneal macrophages ( $5 \times 10^6$ ) were preincubated with 100 ng/ml of *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 24 h and washed twice with culture medium. Cells were stimulated with 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 4 h. Then total RNA was extracted with an RNeasy kit (Qiagen, Hilden, Germany). RNA (10  $\mu$ g) was electrophoresed, transferred to nylon membrane (Hybond N<sup>+</sup>; Amersham Pharmacia Biotech, Uppsala, Sweden) and hybridized with a specific cDNA probe for mouse TNF- $\alpha$ . The same membrane was stripped and rehybridized with a GAPDH cDNA probe for an internal control.

### RT-PCR analysis

Total RNA was extracted with an RNeasy kit (Qiagen) and reverse transcribed using Superscript II (Life Technologies). The cDNA products were PCR amplified with the gene-specific primers. The primer sequences are available upon request.

### Western blot analysis

Peritoneal macrophages were lysed in the lysis buffer containing 1.0% Nonidet P-40, 150 mM NaCl, 10 mM Tris-Cl (pH 7.5), and 1 mM EDTA. The cell lysates were dissolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with the indicated Abs and visualized with an enhanced chemiluminescence system.

### Flow cytometric analysis

Peritoneal macrophages ( $2 \times 10^6$ ) were cultured with 1  $\mu$ g/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 24 h or were not treated. Then cells were harvested and stained with PE-conjugated anti-mouse CD14 Ab or biotin-conjugated MTS510 Ab followed by streptavidin-PE. Stained cells were analyzed on FACScalibur using CellQuest software (Becton Dickinson, Lincoln Park, NJ).

### EMSA and in vitro kinase assay

Peritoneal macrophages ( $2 \times 10^6$ ) were incubated with 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 24 h and washed twice with HBSS. Cells were cultured with culture medium alone for 1 h and then stimulated with 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 10 or 20 min. EMSA and in vitro kinase assay were performed as described previously (18).

## Results

### Synergistic action of LPS and MALP-2 on TNF- $\alpha$ production

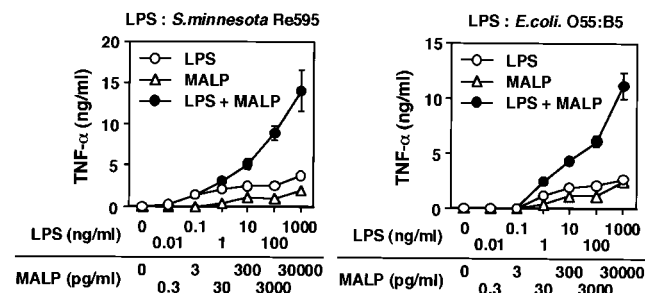
We first examined whether LPS and MALP-2 act synergistically on mouse peritoneal macrophages for induction of cytokine production. Peritoneal macrophages were stimulated with *S. minne-*

*sota* Re595 LPS and/or MALP-2 for 24 h, and then the TNF- $\alpha$  concentration in the culture supernatants of these cells was measured by ELISA (Fig. 1, *left*). Stimulation with LPS or MALP-2 induced TNF- $\alpha$  production from the macrophages, and this production reached a plateau (2.5 or 1 ng/ml) at concentrations of 10 or 0.3 ng/ml, respectively. When cells were stimulated with combinations of 10 ng/ml LPS and 0.3 ng/ml MALP-2, the TNF- $\alpha$  concentration was increased to 5 ng/ml. In addition, when we stimulated with higher concentrations of both LPS and MALP-2, TNF- $\alpha$  production was increased in a dose-dependent manner. The similar synergistic action of MALP-2 and LPS was also observed when we used *E. coli* O55:B5 LPS (Fig. 1, *right*). Thus, when peritoneal macrophages were exposed to LPS and MALP-2 at the same time, they showed synergistic action on cells to produce TNF- $\alpha$ .

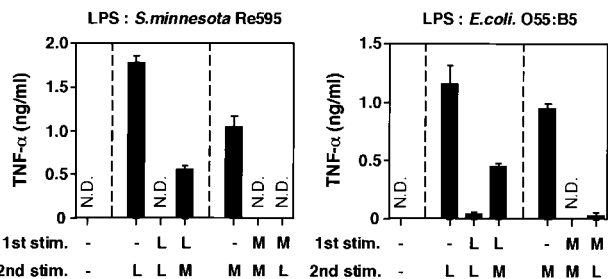
### MALP-2 treatment induces tolerance to the second stimulation with LPS as well as MALP-2

Monocytes/macrophages pre-exposed to LPS show a hyporesponsive state to LPS, which is known as LPS tolerance. Treatment with both *S. minnesota* Re595 LPS and *E. coli* O55:B5 LPS induced tolerance to the second stimulation with LPS (Fig. 2). Several reports indicated that the similar tolerance could be induced by other bacterial cell components, such as peptidoglycan and lipoproteins. We next examined whether MALP-2 has a potential to induce tolerance. When peritoneal macrophages were pre-exposed to MALP-2 for 24 h, a second stimulation with MALP-2 did not induce TNF- $\alpha$  production (Fig. 2). Thus, like LPS tolerance, MALP-2 stimulation induced MALP-2 tolerance in mouse peritoneal macrophages. We next analyzed whether MALP-2 treatment induced tolerance to LPS and vice versa. MALP-2 pretreatment resulted in a significant decrease in TNF- $\alpha$  production in response to LPS, indicating that MALP-2 treatment induced tolerance to LPS. In contrast, when macrophages were pretreated with LPS, these cells produced a significant level of TNF- $\alpha$  in response to MALP-2, although the TNF- $\alpha$  level was somewhat decreased compared with that of nontreated cells. These indicate that LPS-induced tolerance to MALP-2 was weaker than LPS tolerance induced by MALP-2.

We further analyzed TNF- $\alpha$  mRNA expression in response to MALP-2 and LPS by Northern blot analysis (Fig. 3A). Induction of TNF- $\alpha$  mRNA in response to MALP-2 or LPS was significantly reduced in MALP-2- and LPS-pretreated macrophages, respectively. Thus, MALP-2 and LPS tolerance was observed at the level of mRNA expression. We next analyzed cross-tolerance to



**FIGURE 1.** MALP-2 acts synergistically with LPS for the induction of TNF- $\alpha$ . Mouse peritoneal macrophages were stimulated with the indicated concentrations of LPS (*S. minnesota* Re595 or *E. coli* O55:B5) and/or MALP-2 for 24 h. Then the TNF- $\alpha$  concentration in the culture supernatants was assessed by ELISA. The data are the average of three independent experiments.



**FIGURE 2.** MALP-2 pretreatment suppressed TNF- $\alpha$  production in response to both LPS and MALP-2. Peritoneal macrophages were incubated in the presence of 100 ng/ml LPS (L) or 3 ng/ml MALP-2 (M) for 24 h. Then the cells were washed twice and stimulated with 100 ng/ml LPS or 3 ng/ml MALP-2 for an additional 24 h. After the second stimulation, the TNF- $\alpha$  concentration in the culture supernatants was measured by ELISA. The data are the average of three independent experiments. N.D., Not detected.

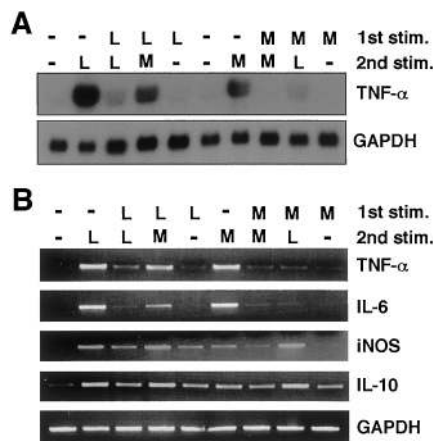
MALP-2 and LPS. In accordance with the TNF- $\alpha$  production determined by ELISA, LPS-induced TNF- $\alpha$  mRNA expression was significantly reduced in MALP-2-pretreated macrophages. In contrast, MALP-2-induced expression was observed in LPS-pretreated macrophages, although it was reduced compared with that in nontreated cells (Fig. 3A). These results further indicate that MALP-2-induced tolerance to LPS was more potent than LPS-induced tolerance to MALP-2.

We also explored RT-PCR to determine induction of mRNA for several genes in tolerant cells (Fig. 3B). Results of RT-PCR analysis for TNF- $\alpha$  and IL-6 mRNA expression showed the same pattern as those of Northern blot and ELISA; that is, LPS did not, but MALP-2 did, induce TNF- $\alpha$  and IL-6 mRNA expression in LPS-pretreated cells. In MALP-2 pretreated cells, neither MALP-2 nor LPS induced these mRNA expressions. Thus, RT-PCR analysis indicated that the similar cross-tolerance occurred in IL-6 induction.

Several reports indicated that the induction of NO and IL-10 was not affected in LPS tolerance (1, 19, 20). We analyzed mRNA expression of inducible NO synthase (iNOS) and IL-10 by RT-PCR (Fig. 3B). Basal expression of both iNOS and IL-10 mRNA was up-regulated in LPS-pretreated cells. However, MALP-2 stimulation led to a further increase in the expression of both iNOS and IL-10 mRNA, but LPS did not. In MALP-2-pretreated cells, expression of both mRNA was slightly up-regulated. The second stimulation with MALP-2 did not increase mRNA expression; however, the second stimulation with LPS did increase it in MALP-2-pretreated cells. Thus, in the case of iNOS and IL-10 induction, the LPS tolerance was observed in LPS-pretreated cells despite the fact that basal mRNA expression was increased, and whereas MALP-2 tolerance was observed in MALP-2-pretreated cells, LPS tolerance was not observed, unlike in induction of TNF- $\alpha$  and IL-6.

#### MALP-2 pretreatment affects LPS-mediated signaling pathway

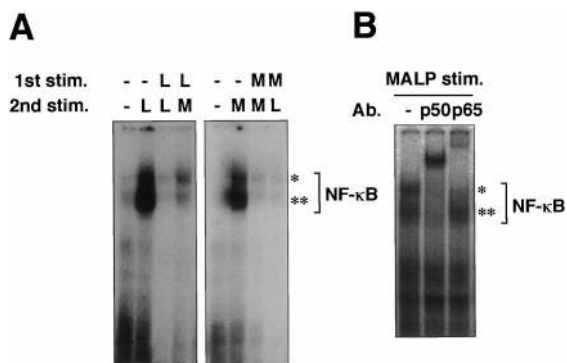
The signaling pathways of both LPS and MALP-2 have been shown to depend on MyD88, leading to activation of NF- $\kappa$ B and JNK (12, 13). We analyzed activation of NF- $\kappa$ B and JNK in MALP-2- and LPS-pretreated macrophages. We first investigated NF- $\kappa$ B activation in response to MALP-2 and LPS (Fig. 4A). Peritoneal macrophages pretreated with MALP-2 or LPS for 24 h were stimulated with 100 ng/ml of *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 20 min. Nuclear extracts from these cells were analyzed with EMSA using a specific probe containing NF- $\kappa$ B binding motif. In nontreated cells, both MALP-2 and LPS stimulation



**FIGURE 3.** MALP-2 pretreatment suppressed mRNA expression of TNF- $\alpha$  in response to both LPS and MALP-2. *A*, Peritoneal macrophages were preincubated with 100 ng/ml *S. minnesota* Re595 LPS (L) or 3 ng/ml MALP-2 (M) for 24 h and then stimulated with 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 4 h. After the second stimulation, total RNA was extracted and subjected to Northern blot analysis using mouse TNF- $\alpha$  cDNA probe. The same membrane was rehybridized with a mouse GAPDH cDNA probe. Similar results were obtained from three independent experiments. *B*, Total RNA was reverse transcribed and PCR amplified using the indicated primers.

induced a significant increase in NF- $\kappa$ B DNA binding activity. Both MALP-2 and LPS stimulation resulted in the formation of two major DNA-protein complexes (\* and \*\* in Fig. 4A). To determine which subunits of NF- $\kappa$ B comprised these two complexes, we conducted supershift experiments using Abs to p50 and p65 subunit of NF- $\kappa$ B (Fig. 4B). Preincubation with anti-p50 Ab resulted in supershifts of both upper (\*) and lower (\*\*) bands. In contrast, anti-p65 Ab only induced supershift of upper band. These demonstrate that the upper band represents p50/p65 heterodimer, and the lower band represents p50/p50 homodimer. When cells were pretreated with LPS, augmentation of NF- $\kappa$ B activity in response to second stimulation with LPS was almost completely suppressed (Fig. 4A). Likewise, MALP-2-induced NF- $\kappa$ B activation was not observed in MALP-2-pretreated cells. Thus, NF- $\kappa$ B activity was severely reduced in both MALP-2 and LPS tolerance. In MALP-2-pretreated cells, not only MALP-2-induced but also LPS-induced augmentation of NF- $\kappa$ B activity were severely reduced, indicating that MALP-2 treatment affected both MALP-2- and LPS-mediated signaling pathways. In contrast, MALP-2 stimulation significantly increased NF- $\kappa$ B activity in LPS-pretreated cells; p50/p65 heterodimers (*upper band*, \*) was mainly induced (Fig. 4A) Thus, MALP-2 treatment affected both MALP-2- and LPS-mediated NF- $\kappa$ B activity; in contrast, LPS treatment affected LPS-mediated, but less effectively MALP-2-mediated, NF- $\kappa$ B activity in macrophages.

Then MALP-2- or LPS-induced JNK activation was analyzed by *in vitro* kinase assay (Fig. 5A). JNK was activated in response to both LPS and MALP-2 in nontreated cells. JNK activation was almost completely diminished when cells were pretreated with the same stimulant. Furthermore, LPS-induced JNK activation was not observed in MALP-2-pretreated cells; in contrast, MALP-2-induced JNK activation was significantly observed, although at a reduced level, in LPS-pretreated cells. Taken together, these results indicate that signaling pathways of both MALP-2 and LPS were affected in MALP-2-pretreated macrophages; in contrast, the LPS-mediated, but not the MALP-2-mediated, signaling pathway was affected in LPS-pretreated cells.



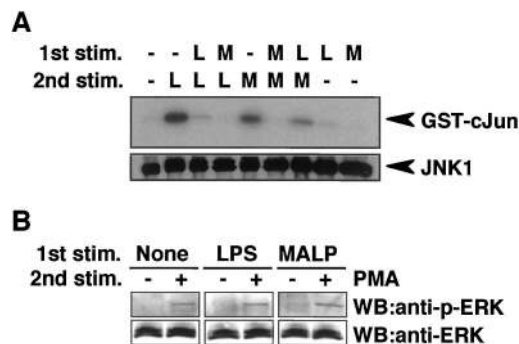
**FIGURE 4.** Altered NF- $\kappa$ B activation in MALP-2- or LPS-pretreated macrophages. Peritoneal macrophages were cultured with 100 ng/ml of *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 24 h or were not treated. Cells were washed twice and then stimulated with 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 20 min. After the second stimulation, nuclear extracts were extracted and incubated with a specific probe containing NF- $\kappa$ B binding sites, and NF- $\kappa$ B activity was determined by gel mobility shift assay (A). The specificity of shifted bands (\* and \*\*) was determined by adding specific Abs to p50 or p65 (B). Similar results were obtained from four independent experiments.

LPS-tolerant macrophages have been shown to display a normal response to an unrelated stimulation such as PMA (17, 21). Therefore, we investigated PMA-induced activation of mitogen-activated protein kinase (ERK-1 and 2) in LPS- and MALP-2-pretreated macrophages (Fig. 5B). In LPS- or MALP-2-pretreated cells, PMA-induced phosphorylation of ERK-1 and 2 was observed at a level similar to that in nontreated cells. Thus, LPS- or MALP-2-pretreated cells showed no impaired response to an unrelated stimulation.

#### Surface expression of the TLR4-MD2 complex was not reduced by MALP-2 stimulation

We next addressed expression of several components involved in the signaling pathways for LPS and MALP-2. RT-PCR analysis showed that mRNA expression of genes, such as TLR4, TLR2, MD2, MyD88, and IRAK-1, was not reduced in LPS- and MALP-2-treated macrophages (Fig. 6A). Western blot analysis further demonstrated that expression of IRAK-1 was not reduced in LPS- and MALP-2-treated cells (Fig. 6B).

We have recently demonstrated that LPS stimulation resulted in the reduced surface expression of the TLR4-MD2 complex on peritoneal macrophages, and this phenomenon might account for LPS tolerance (16). Our present data demonstrate that MALP-2 pretreatment induced tolerance to LPS, as is the case in LPS tolerance. Therefore, we next investigated surface expression of the TLR4-MD2 on MALP-2-treated macrophages. Cells were stimulated with *S. minnesota* Re595 LPS or MALP-2 for 24 h, then stained with Ab that recognizes the TLR4-MD2 complex (17). When macrophages were stimulated with LPS, surface expression of the TLR4-MD2 complex was severely reduced (Fig. 6C, left). However, the surface expression of the TLR4-MD2 complex was not reduced, rather it was enhanced, in MALP-2-treated macrophages. CD14 is another surface molecule responsible for the recognition of LPS. MALP-2 stimulation did not reduce surface CD14 expression, as is the case in LPS stimulation (Fig. 6C, right). These results suggest that MALP-2-induced tolerance to LPS did not occur through down-regulation of the surface TLR4-MD2 complex and CD14 expression, indicating that MALP-2-induced tolerance occurs through mechanisms other than LPS-induced tolerance.



**FIGURE 5.** Reduced activation of JNK in MALP-2- or LPS-treated macrophages. A, Peritoneal macrophages pretreated with 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 24 h were stimulated with 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 10 min. After the second stimulation, cell lysates were prepared and immunoprecipitated with anti-JNK1 Ab. The activity of JNK in the immunoprecipitates was measured by an in vitro kinase assay using GST-c-Jun fusion protein as a substrate (upper panel). The same lysates were blotted with anti-JNK1 Ab to monitor the expression of JNK1 (lower panel). Similar results were obtained from three independent experiments. B, Peritoneal macrophages pretreated with LPS or MALP-2 were stimulated with 50 ng/ml PMA for 15 min. Cells were lysed and subjected to Western blot analysis using anti-phospho-ERK-1 and 2 Ab.

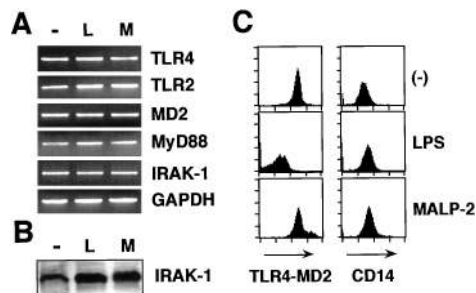
#### LPS- and MALP-2-induced tolerance was not mediated by IL-10

In LPS- and MALP-2-pretreated macrophages, IL-10 mRNA was constitutively expressed (Fig. 3B). IL-10 is known to suppress macrophage activity. Although LPS tolerance has been shown to be induced independently of IL-10, there remains the possibility that MALP-2-induced tolerance is dependent on IL-10 (22). Therefore, we analyzed whether IL-10 was involved in LPS- and MALP-2-induced tolerance using IL-10-deficient mice. Peritoneal macrophages from wild-type and IL-10-deficient mice were pretreated with LPS or MALP-2, then restimulated and analyzed for TNF- $\alpha$  production by ELISA (Fig. 7). In LPS-pretreated IL-10-deficient macrophages, LPS did not but MALP-2 did induce TNF- $\alpha$  production. In MALP-2-pretreated IL-10-deficient macrophages, neither MALP-2 nor LPS induced TNF- $\alpha$  production. Thus, macrophages from IL-10-deficient mice displayed the similar cross-tolerance state as wild-type mice, indicating that MALP-2-induced tolerance occurs independently of IL-10 induction.

#### Discussion

In the present study, we have examined the effect of bacterial cell wall components that are differentially recognized by distinct TLR family members on mouse peritoneal macrophages. We used MALP-2 and LPS as bacterial cell components, because the analyses of gene-targeted mice clearly demonstrated that TLR2 is an essential pattern recognition receptor for MALP-2, just as TLR4 is for LPS (11, 13).

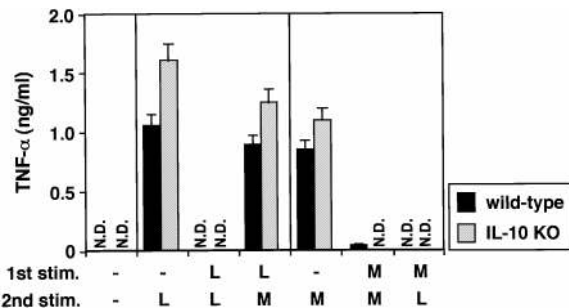
We first demonstrated that MALP-2 and LPS synergistically act on peritoneal macrophages and induce production of inflammatory cytokines. An adaptor molecule, MyD88, is involved in the signaling pathway via TLR4 (19, 20). Upon stimulation, MyD88, which binds to TLR4, recruits IRAK to the receptor. IRAK then activates TRAF6, leading to activation of NF- $\kappa$ B and JNK (23, 24). Indeed, both MyD88- and TRAF6-deficient mice displayed hyporesponsiveness to LPS (18, 25, 26). MyD88-deficient mice especially were almost completely unresponsive to LPS, indicating that the MyD88-dependent pathway is essential for inflammatory responses to LPS (18). Furthermore, MyD88-deficient mice



**FIGURE 6.** Surface expression of TLR4 on MALP-2- or LPS-treated peritoneal macrophages. *A*, Peritoneal macrophages were cultured for 24 h in the presence of 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2. Total RNA was extracted, reverse transcribed, and PCR amplified with the indicated primers. *B*, Peritoneal macrophages treated with 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 24 h was analyzed for IRAK-1 expression by Western blot analysis. *C*, Peritoneal macrophages were stimulated with 1  $\mu$ g/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2 for 24 h. Then cells were incubated with biotinylated MTS510 Abs, followed by PE-conjugated streptavidin or PE-conjugated anti-CD14 Ab and analyzed by flow cytometry. Similar results were obtained from three independent experiments.

showed no inflammatory response to several other bacterial components, including peptidoglycan and MALP-2 (13, 27). Both peptidoglycan and MALP-2 have been shown to be recognized by TLR2. Thus, MyD88 is the adaptor molecule shared by TLR2- and TLR4-mediated signaling pathways. Although the molecular mechanism of the synergistic action of MALP-2 and LPS is unknown, our present study indicates that the simultaneous activation of different TLRs could exert the synergistic effects. Indeed, bacterial lipopeptides and lipoprotein have been shown to induce inflammatory cytokine production in synergy with LPS (4, 28). The mycoplasmal lipopeptide MALP-2 is structurally related to bacterial lipopeptides and lipoprotein, all of which have been shown recognized by TLR2 (29–33). Several other reports demonstrated that bacterial DNA also synergistically acts with LPS for induction of inflammatory cytokine production (34–36). A responsible pattern recognition receptor of bacterial DNA has not been identified. However, when we consider that bacterial DNA and LPS synergistically act in a manner similar to MALP-2 and LPS, we can hypothesize that bacterial DNA is recognized by a member of the TLR family other than TLR4.

We next addressed the cross-tolerance between MALP-2 and LPS. MALP-2 induced tolerance to the second stimulation with MALP-2, like LPS tolerance. In addition, when macrophages were treated with MALP-2, these cells showed the reduced TNF- $\alpha$  production in response to the second LPS stimulation, demonstrating that MALP-2 treatment induced tolerance to LPS as well as to MALP-2. Our previous study demonstrated that a major cause of LPS tolerance is the down-regulation of the TLR4-MD2 complex after LPS pretreatment. Reduction of surface expression of the TLR4-MD2 complex was observed even in C3H/HeJ mice, which are hyporesponsive to LPS (16). The rapid down-regulation of TLR4-MD2 might occur by an internalization of LPS along with the receptor complex. This hypothesis was supported by the recent report demonstrating that surface TLR2 on macrophages was rapidly internalized to phagosome after treatment with zymosan, a component of the yeast cell wall (37). Therefore, in the MALP-2-treated macrophages, surface TLR2 might be down-regulated, as is the case in zymosan-treated macrophages. However, we cannot explain the mechanism of the MALP-2-induced tolerance to LPS by down-regulation of surface TLR2 expression, because TLR2-deficient mice showed the normal response to LPS (12). Further-



**FIGURE 7.** Cross-tolerance was induced in an IL-10-independent manner. Peritoneal macrophages from IL-10-deficient or wild-type mice were cultured for 24 h in the presence of 100 ng/ml *S. minnesota* Re595 LPS or 3 ng/ml MALP-2. Then the cells were washed twice and stimulated with 100 ng/ml LPS or 3 ng/ml MALP-2 for an additional 24 h. After the second stimulation, the TNF- $\alpha$  concentration in the culture supernatants was measured by ELISA. N.D., Not detected.

more, macrophages treated with MALP-2 did not show any decreased expression of the surface TLR4-MD2 complex. Despite the normal TLR4 expression in MALP-2-treated macrophages, LPS-induced NF- $\kappa$ B and JNK activation was severely reduced. These findings indicate that MALP-2-induced tolerance to LPS is due to the affected LPS signaling pathway, but not to the reduced surface expression of the TLR4-MD2 complex. Interestingly, when macrophages were pretreated with LPS, induction of tolerance to MALP-2 was poor. Production of TNF- $\alpha$  as well as activation of NF- $\kappa$ B and JNK in response to MALP-2 were significantly observed in LPS-pretreated macrophages. A similar phenomenon has been reported in several cross-tolerance models. Pretreatment with bacterial DNA induced LPS tolerance; however, LPS pretreatment was less effective in induction of tolerance to bacterial DNA (34, 38). Likewise, although muramyl dipeptide pretreatment significantly reduced the serum level of inflammatory cytokines after administration of LPS, LPS treatment did not reduce inflammatory cytokine production after muramyl dipeptide administration in guinea pig (2). Thus, LPS treatment has been shown to be less effective in induction of tolerance to other bacterial components. In this point our speculation is as follows. In both LPS and MALP-2 pretreatment, tolerance occurs through down-regulation of surface TLRs as well as inhibition of the downstream signaling pathways. In the case of MALP-2 pretreatment, the shut-off of the downstream signaling pathway takes place at a similar pace with the down-regulation of TLR2. In contrast, when pretreated with LPS, the down-regulation of surface TLR4 is dominant, and thereby the downstream signaling pathway is not severely affected.

In LPS-pretreated macrophages, mRNA expression of iNOS and IL-10 was constitutively observed, but subsequent LPS stimulation did not enhance the mRNA expression, indicating that the cells were tolerant to LPS. Several previous studies reported that production of NO and IL-10 was induced by LPS even in the LPS-tolerant cells (19, 20). These data seem contradictory to our findings; however, in these papers macrophages were pretreated with a low concentration of LPS (<20 ng/ml). We previously reported that induction of LPS tolerance was not severe when stimulated with a low concentration of LPS (16). Thus, the low concentration of LPS might not effectively induce LPS tolerance.

In MALP-2-pretreated cells, LPS-induced expression of iNOS and IL-10 was not severely affected. In this regard we speculate as follows. MALP-2 pretreatment affects mainly the TLR-MyD88-dependent signaling pathway, which is essential for LPS-induced TNF- $\alpha$  induction, as demonstrated in MyD88-deficient mice (18).

However, even in MyD88-deficient mice, LPS-induced activation of the signaling cascades and expression of several genes other than TNF- $\alpha$  and IL-6 were observed (our unpublished observations). These indicate that an unidentified MyD88-independent signaling pathway(s) does exist. In MALP-2-pretreated macrophages, surface expression of the TLR4-MD2 complex was not reduced, which means that possibly LPS-induced activation of the MyD88-independent signaling pathway occurs. Therefore, we suspect that although LPS-induced activation of the MyD88-dependent signaling pathway was affected, activation of the MyD88-independent signaling pathway in MALP-2-pretreated cells might account for the induction of iNOS and IL-10. Elucidation of precise mechanisms of tolerance induction will require additional experiments.

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