Cutting Edge: Endotoxin Tolerance in Mouse Peritoneal Macrophages Correlates with Down-Regulation of Surface Toll-Like Receptor 4 Expression

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Monocytes/macrophages exposed to LPS show reduced responses to second stimulation with LPS, which is termed LPS tolerance. In this study, we investigated molecular mechanisms of LPS tolerance in macrophages. Mouse peritoneal macrophages pre-exposed to LPS exhibited reduced production of inflammatory cytokines in a time- and dose-dependent manner. Activation of neither IL-1 receptor-associated kinase nor NF-κB was observed in macrophages that became tolerant by LPS pretreatment, indicating that the proximal event in Toll-like receptor 4 (TLR4)-MyD88-dependent signaling is affected in tolerant macrophages. Although TLR4 mRNA expression significantly decreased within a few hours of LPS pretreatment and returned to the original level at 24 h, the surface TLR4 expression began to decrease within 1 h, with a gradual decrease after that, and remained suppressed over 24 h. A decrease in inflammatory cytokine production in tolerant macrophages well correlates with down-regulation of the surface TLR4 expression, which may explain one of the mechanisms for LPS tolerance. The Journal of Immunology, 2000, 164: 3476–3479.

Lipopolysaccharide, a major cell wall component of Gram-negative bacteria, induces activation of monocytes and macrophages. Activated macrophages produce several inflammatory cytokines including TNF-α, IL-6, and IL-12, which, when in excess, leads to serious systemic disorders with a high mortality rate. Pre-exposure to LPS is shown to induce a reduced sensitivity to subsequent challenge of LPS. This phenomenon is termed LPS tolerance (also called LPS hyporesponsiveness or refractoriness). LPS tolerance was observed in vivo with a decreased febrile response and an escape from lethality as well as in vitro with a reduced production of inflammatory cytokines in response to a secondary stimulation with LPS.

Molecular mechanisms for LPS tolerance have long been investigated (1). Several reports described that expression of the LPS receptor such as CD14 was not altered in LPS-tolerant monocytes and macrophages (2, 3). In contrast, LPS-signaling pathways have been shown to be affected in several aspects. It has been reported that activation of proteins kinase C was compromised in LPS-tolerant macrophages (4). Several other reports demonstrated that there is a predominant accumulation of p50/p50 homodimers of NF-κB transcription factors in LPS-tolerant cells (3, 5, 6). In normal cells, NF-κB mainly consists of p50/p65 heterodimers, and this complex induces expression of target genes. On the other hand, p50/p50 homodimers do not have transactivation property and prevent DNA binding of p50/p65 heterodimers. Thus, it is hypothesized that LPS tolerance occurs through alteration of the intracellular signaling pathways of LPS. However, a precise mechanism for LPS tolerance remains unclear.

It has recently been demonstrated that the genetically LPS-hyporesponsive C3H/HeJ mice has a mutation in the Tlr4 gene (7, 8). Toll-like receptor 4 (TLR4) is a member of the Toll-like receptor family, which is an expanding large family in mammals (9–12). *Drosophila* Toll has been shown to have a critical role in antifungal and antibacterial responses (13–16). Several recent reports have also demonstrated that Toll-like receptors are involved in recognition of bacterial cell wall components (8, 17–23). TLR4-deficient mice showed hyporesponsiveness to LPS, demonstrating...
that TLR4 is a critical receptor for LPS signaling (20). The TLR4-mediated signaling pathway is homologous to that of IL-1 signaling (10, 24, 25). An adaptor molecule MyD88 binds to TLR4. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLR4. IRAK then activates TNFR-associated factor 6 (TRAF6), leading to activation of NF-κB and c-Jun N-terminal kinase. Indeed, MyD88- and TRAF6-deficient mice displayed hyporesponsiveness to both IL-1 and LPS (26, 27). Especially, MyD88-deficient mice are almost completely unresponsive to LPS (26). Thus, analyses of gene-targeted mice demonstrate that the pathway via TLR4-MyD88 is essential for LPS response.

In this study, we investigated whether the pathway via TLR4-MyD88 is involved in LPS tolerance. We show that TLR4 expression on the surface of LPS-tolerant macrophages is down-regulated, which explains one of the molecular mechanisms for LPS tolerance.

Materials and Methods

Cells and reagents

Peritoneal macrophages were isolated from C57BL/6J mice essentially as described. Briefly, mice were i.p. injected with 2 ml of 4% thioglycollate. 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.

Phenol-extracted LPS (Escherichia coli O55:B5) was purchased from Sigma (St. Louis, MO). PE-conjugated Abs to IL-6 and IL-12 were purchased from PharMingen (San Diego, CA).

Intracellular staining of macrophages

Peritoneal macrophages were preincubated with 1, 10, or 100 ng/ml LPS for the indicated periods and washed with HBSS twice. Cells were stimulated with 10 ng/ml LPS in the presence of 10 μg/ml brefeldin A (Sigma) for 6 h. Cells were harvested and incubated with 4% paraformaldehyde. Then cells were incubated with PE-conjugated anti-cytokine Abs. Stained cells were analyzed on a FACSCalibur using CellQuest software (Becton Dickinson, San Jose, CA).

Electrophoretic mobility shift assay and in vitro kinase assay

Peritoneal macrophages were incubated with 100 ng/ml LPS for the indicated periods and washed with HBSS. Cells were cultured with culture media alone for 1 h and then stimulated with 10 ng/ml LPS for 10 or 20 min. An electrophoretic mobility shift assay and in vitro kinase assay were performed as described previously (26).

Northern blot analysis

Peritoneal macrophages and RAW264.7 cells were incubated with 100 ng/ml LPS for the indicated periods. Total RNA was extracted with an RNeasy kit (Qiagen, Hilden, Germany). RNA (20 μg) was electrophoresed, transferred to nylon membrane, and hybridized with cDNA probe for mouse TLR4. The same membrane was stripped and rehybridized with GAPDH cDNA probe.

Establishment of a mAb to mouse TLR4

A rat was immunized with Ba/F3 cells expressing mouse TLR4 and MD-2 and used for hybridoma production. The MTS 510 mAb (rat IgG2a/k) that specifically reacted with the immunized transfected but not with the original Ba/F3 line was selected for further analysis. The mAb was purified from ascites obtained from severe combined immunodeficient mice. Detailed characterization of the mAb will be described elsewhere (29).

Results and Discussion

Time-dependent and dose-dependent suppression of inflammatory cytokine production by pre-exposure to LPS

When mouse peritoneal macrophages were stimulated with 10 ng/ml LPS, these cells displayed a significant increase in production of inflammatory cytokines such as IL-12 and IL-6 (Fig. 1). However, when the cells were preincubated with 100 ng/ml LPS for 1 h, IL-12 production was dramatically reduced (30.6 to 4.6% positive). When the cells were preincubated for 24 h, IL-12 production was almost completely blocked. Pre-exposure to LPS for 1 h also partially reduced production of IL-6 (15.1 to 8.8%). In addition, production of IL-6 reduced with the lapse of pre-exposure time, and production was severely reduced after 24 h of pre-exposure (0.8%). Thus, suppression of inflammatory cytokine production from LPS-pretreated macrophages was observed in a time-dependent manner.

We further preincubated peritoneal macrophages with several doses of LPS for 24 h and analyzed production of inflammatory cytokines. As shown in Fig. 1, 100 ng/ml LPS dramatically reduced the production of IL-12 and IL-6. However, when preincubated with 10 ng/ml, the reduction was partial, and further 1 ng/ml LPS did not cause significant reduction (Fig. 1). These results suggest that suppression of inflammatory cytokine production was observed in a dose-dependent manner. Thus, when we used a system to detect intracellular cytokines, LPS tolerance does occur in a time-dependent and a dose-dependent manner.

Reduced activation of LPS-signaling cascade after exposure to LPS

The results from intracellular cytokine production indicate that a 24-h exposure to LPS results in almost complete LPS tolerance, but it is partial after a 3-h exposure. To assess LPS-induced activation of signaling molecules during LPS tolerance, we analyzed LPS-induced NF-κB activation by gel mobility shift assay (Fig. 2A). In nontreated cells, DNA-binding activity of NF-κB transcription factors was slightly observed, and LPS stimulation induced a significant increase in their DNA-binding activity. When pretreated with LPS for 3 h, basal NF-κB activity was still observed; however, LPS-induced increase was not observed. In the...
24-h pretreated cells, neither basal NF-κB activity nor LPS-induced activation was observed. Thus, LPS pre-exposure significantly reduced NF-κB DNA-binding activity.

IRAK is known to be a downstream kinase of MyD88, which acts as an adaptor molecule in the LPS-signaling pathway (26). Stimulation with LPS for 10 min induced phosphorylation of IRAK in nontreated macrophages (Fig. 2B). However, IRAK activation was not observed in cells pre-exposed to LPS for 3 and 24 h. Thus, LPS-induced IRAK activation was severely reduced in cells pre-exposed to LPS. We have previously shown that LPS-induced IRAK activation was not observed in both MyD88-deficient and TLR4-deficient mice (22, 26). Both strains of knockout mice are unresponsive to LPS. TLR4 knockout mice also displayed no LPS-induced NF-κB activation. These findings are quite similar to the data in the macrophages pre-exposed to LPS for 24 h. Therefore, we hypothesized that tolerant macrophages are affected in the MyD88-dependent pathway, which is essential for LPS responsiveness.

Decreased expression of TLR4 after exposure to LPS

We analyzed expression of TLR4, an essential signaling receptor for LPS. The former study demonstrated that LPS stimulation transiently reduced mRNA expression of TLR4 in the macrophage cell line RAW264.7 (7). We also obtained similar results in RAW264.7 cells. When cells were stimulated with 100 ng/ml LPS for 2.5 h, TLR4 mRNA expression was severely reduced; however, the expression returned to the original level after a 20-h stimulation (Fig. 3A). When mouse peritoneal macrophages were stimulated with 100 ng/ml LPS, TLR4 mRNA expression was also transiently suppressed (Fig. 3B). In both types of cells, TLR4 mRNA expression at 24-h LPS treatment was almost the same level as that of nontreated cells. Thus, the mRNA expression pattern of TLR4 seems not to correlate with LPS tolerance.

We next analyzed surface expression of TLR4 using recently generated mAb MTS510. MTS510 was shown to detect the complex of murine TLR4 and MD-2 and inhibit LPS-induced NF-κB activation and TNF production (28, 29). Almost all nontreated macrophages were positive for this Ab (Fig. 4A). The specificity of this Ab was confirmed by the staining of macrophages from TLR4 knockout mice. In TLR4 knockout mice, macrophages were negatively stained with this Ab, indicating that a positive staining by...
 Interestingly, the nuclear extract from the macrophages pretreated must be some modifications of intracellular transport or stability of solely due to suppression of the surface expression of TLR4. There into the culture supernatants in these periods was not observed (our blocked during 1–12-h pre-exposure, secretion of these cytokines ERANCE than down-regulation of TLR4 expression. Although intra-macrophages correlates with suppression of the surface TLR4 expression. The LPS-signaling pathway has been intensively investigated for a long time and recently remarkable progress has been made. MyD88 and TRAF6 are shown to be its key signaling mole-
ules (26, 27). However, a molecular mechanism for LPS tolerance (7, 8, 20). MyD88-dependent signaling pathway (7, 8, 20). These observa-
tsions indicate that down-regulation of TLR4 expression occurs in-
dependently of the MyD88-dependent signaling pathway. However, there seems to exist other mechanisms for LPS tol-
erance than down-regulation of TLR4 expression. Although intra-
cellular production of inflammatory cytokines was not completely blocked during 1–12-h pre-exposure, secretion of these cytokines into the culture supernatants in these periods was not observed (our unpublished data). This suggests that LPS tolerance does not occur solely due to suppression of the surface expression of TLR4. There must be some modifications of intracellular transport or stability of newly synthesized proteins in the LPS-tolerant macrophages. Inter-
estingly, the nuclear extract from the macrophages pretreated for 24 h did not show the basal NF-κB-binding activity that is observed in the nuclear extract from untreated macrophages. Thus, although down-regulation of TLR4 expression is responsible for LPS tolerance, other mechanisms along with it seem to operate toward LPS tolerance during LPS pretreatment.

Acknowledgments

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