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

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

Fumiko Nomura,*†§ Sachiko Akashi,‡ Yoshimitsu Sakao,*§ Shintaro Sato,*§ Taro Kawai,*§ Makoto Matsumoto,*§ Kenji Nakanishi,†§ Masao Kimoto,‡ Kensuke Miyake,‡ Kiyoshi Takeda,*§ and Shizuo Akira*‡§

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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). 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-tolerant macrophages is down-regulated, which explains one of the molecular mechanisms for LPS-tolerant macrophages is down-regulated, which explains one of the molecular mechanisms for...

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 cultured 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 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-tolerant 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 occurs 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 with LPS contains newly synthesized proteins in the LPS-tolerant macrophages. In must be some modifications of intracellular transport or stability of these proteins that solely due to suppression of the surface expression of TLR4. There was not observed (our unpublished data). 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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References

22. Takeuchi, O., T. Kawai, H. Sanjo, T. Kaida, T. Ogawa, K. Takeda, and S. Akira. 1999. Toll-like receptor-4 and peptidoglycan (PGN), both of which activate the MyD88-dependent pathway via other receptors than TLR4. IL-1β and PGN are utilized to the IL-1R and TLR2 as the signaling receptor, respectively (22). Both stimulations for 24 h did not affect surface TLR4 expression, indicating that the reduced expression of surface TLR4 is induced only when macrophages are exposed to LPS, although all of these stimuli activate the same MyD88-dependent pathway (Fig. 4D). Interestingly, reduction of surface TLR4 expression after LPS treatment was observed even in C3H/HeJ mice in a manner similar to that in C3H/HeN mice (Fig. 4E). C3H/HeJ mice are shown to have a mutation in the Tlr4 gene, leading to the impaired LPS-mediated MyD88-dependent signaling pathway (7, 8, 20). These observations indicate that down-regulation of TLR4 expression occurs independently of the MyD88-dependent signaling pathway.

The LPS-signaling pathway has been intensively investigated for a long time and recently remarkable progress has been made. TLR4 is found to be an essential receptor for LPS signaling (7, 8, 20). MyD88 and TRAF6 are shown to be its key signaling molecules (26, 27). However, a molecular mechanism for LPS tolerance remains unclear. In the present study, we present one of the mechanisms for LPS tolerance by demonstrating that LPS tolerance in macrophages correlates with suppression of the surface TLR4 expression. However, there seems to exist other mechanisms for LPS tolerance than down-regulation of TLR4 expression. Although intracellular production of inflammatory cytokines was 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. Interestingly, the nuclear extract from the macrophages pretreated for 24 h did 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.