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Unresponsiveness of MyD88-Deficient Mice to Endotoxin

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Summary

MyD88 is a general adaptor protein that plays an important role in the Toll/IL-1 receptor family signalings. Recently, Toll-like receptors 2 and 4 (TLR2 and TLR4) have been suggested to be the signaling receptors for lipopolysaccharide (LPS). In this study, we demonstrate that MyD88 knockout mice lack the ability to respond to LPS as measured by shock response, B cell proliferative response, and secretion of cytokines by macrophages and embryonic fibroblasts. However, activation of neither NF-κB nor the mitogen-activated protein (MAP) kinase family is abolished in MyD88 knockout mice. These findings demonstrate that signaling via MyD88 is essential for LPS response, but the inability of MyD88 knockout mice to induce LPS-dependent gene expression cannot simply be attributed to lack of the activation of MAP kinases and NF-κB.

Introduction

Lipopolysaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria and a potent activator of macrophage functions (Ulevitch and Tobias, 1995). Stimulation of macrophages with LPS results in the production of various cytokines such as TNFα, IL-1, IL-6, IL-10, macrophage inflammatory protein-1α/β (MIP-1 α/β), and inflammatory effector substances such as protanoids, leukotrienes, and nitric oxide (NO), as well as the enhanced expression of cell surface antigens such as major histocompatibility complex (MHC) class II and B7-1/2 (Ulevitch and Tobias, 1995). Although LPS in appropriate amounts can trigger responses that are favorable to the host by augmenting microbicidal activities of macrophages, overactivation of macrophages by large amounts of LPS results in endotoxin shock. Therefore, the effect of LPS on macrophage activation has been extensively studied, but the molecular mechanisms on macrophage activation and signaling events in response to LPS are still not well understood.

CD14 is a glycosylphosphatidyl inositol (GPI) anchored macrophage cell surface glycoprotein and functions as an essential receptor for LPS in the presence of LPS-binding protein (LBP) (Wright et al., 1990). Subsequent to the occupation of LPS/LBP/CD14 ternary complex on the cell surface, multiple signal transduction pathways are activated, including the activation of nonreceptor tyrosine kinases, protein kinase C, several members of the mitogen-activated protein (MAP) kinase family, and transcriptional factor NF-κB (Weinstein et al., 1991; Dong et al., 1993; Han et al., 1994; Shinji et al., 1994; Hambleton et al., 1996; Sanghera et al., 1996). Although it is LBP and CD14 that primarily recognize LPS (Haziot et al., 1996), several studies have suggested the existence of a coreceptor that initiates signal transduction (Perera et al., 1997).

Toll was initially identified as a transmembrane protein that is involved in establishing the dorsoventral polarity of *Drosophila* embryo and is also involved in the defensive response to fungal infection (Belvin and Anderson, 1996; Lemaitre et al., 1996). There are remarkable structural and functional similarities between the *Drosophila* Toll- and mammalian IL-1R-mediated signalings. The intracellular portion of Toll shares sequence homology with that of the mammalian IL-1 receptor (IL-1R) (Medzhitov and Janeway, 1997; O'Neill and Greene, 1998). Toll activates Dorsal, a *Drosophila* homolog of NF-κB through the degradation of cactus, a homolog of IκB. The *Drosophila* serine/threonine kinase Pelle is highly homologous to the IL-1 receptor associated kinase (IRAK) (Cao et al., 1996a; Muzio et al., 1997). Recently, a human homolog of Toll (human Toll/TLR4) was identified (Medzhitov et al., 1997). Five human Toll-like receptors (TLR1-5) have subsequently been cloned (Chaudhary et al., 1998; Rock et al., 1998). These are an orphan receptor family with an extracellular portion containing leucine-rich repeats and a cytoplasmic domain significantly similar to the intracellular portion of the IL-1R family. Therefore, they were expected to use the same signaling molecules as with IL-1Rs. In fact, signaling through human Toll/TLR4, as shown in IL-1R signaling, occurs through sequential recruitment of the adaptor molecule MyD88 and IRAK (Muzio et al., 1997, 1998; Wesche et al., 1997a; Adachi et al., 1998; Burns et al., 1998; Medzhitov et al., 1998). TRAF6 and NIK are both involved in subsequent steps of NF-κB activation (Cao et al., 1996b; Malinin et al., 1997). Recently, Toll-like receptor 2 (TLR2) has been suggested to be involved in LPS-mediated signaling (Kirschning et al., 1998; Yang et al., 1998). TLR2 binds LPS in the presence of LBP and CD14 and induces NF-κB activation. Dominant-negative TLR2 specifically blocked LPS-mediated NF-κB activation.

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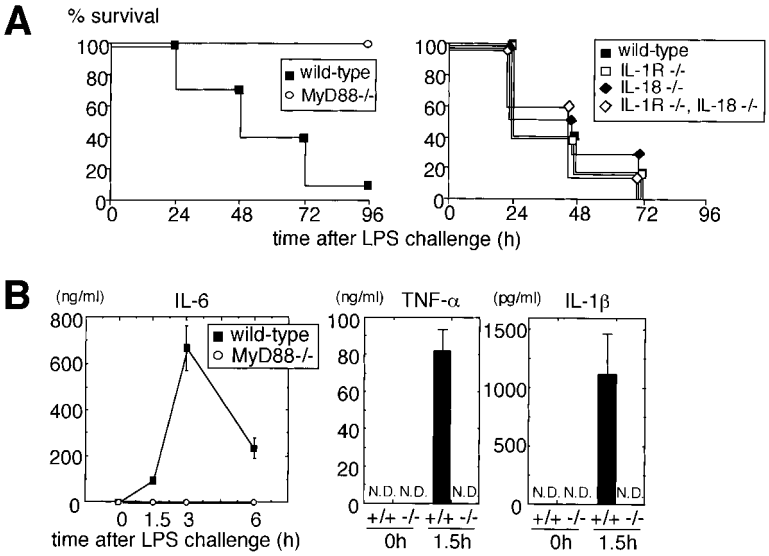

Figure 1. MyD88 KO Mice Are Resistant to LPS-Induced Endotoxin Shock

(A) Age-matched wild-type (n = 8) and MyD88 KO mice (n = 8) were intraperitoneally injected with 1.0 mg of LPS from *E. coli* serotype O55:B5. Survival was monitored for 4 days. Age-matched wild-type (n = 5), IL-18 KO (n = 6), IL-1R KO (n = 5), and IL-18/IL-1R double KO (n = 5) mice were intraperitoneally injected with 1.0 mg of O55:B5 LPS. Survival was monitored for 4 days.

(B) Age-matched wild-type (n = 8) and MyD88 KO (n = 8) mice were injected with 1.0 mg of O55:B5 LPS. Sera were taken at 1.5, 3, and 6 hr after LPS injection. Serum concentrations of IL-6, TNFα, and IL-1β were measured by specific enzyme-linked immunosorbent assay (ELISA). ND, not detected.

More recently, it has been shown that the LPS hyporesponsiveness in C3H/HeJ mouse strain is due to a missense point mutation of the tlr4 gene (Poltorak et al., 1998; Hoshino et al., 1999; Qureshi et al., 1999). Macrophages and B cells from TLR4-deficient mice are hyporesponsive to LPS, indicating that TLR4 is required for LPS signaling. These findings show that the TLR family is actually involved in LPS recognition and signaling in mammals, although the relation between TLR2 and TLR4 in LPS response is still unclear.

In the present study, we examined the role of MyD88 in LPS response. All of the LPS-mediated functions examined were almost completely abolished in MyD88 knockout (KO) mice. These results demonstrate that MyD88 plays a critical role for LPS-mediated functions. Unlike IL-1 and IL-18 signaling, both MAP kinases and NF-κB were activated in response to LPS in MyD88 KO mice, indicating the presence of MyD88-independent pathway(s) in LPS signaling.

Results and Discussion

High Resistance to LPS-Induced Shock in MyD88 KO Mice

We first analyzed LPS responsiveness in MyD88 KO mice. Mice were intraperitoneally injected with high doses of LPS, and their survival was monitored. Almost all wild-type mice were dead within 96 hr after LPS injection. In contrast, MyD88 KO mice showed a high resistance to LPS-induced shock (Figure 1A). MyD88 has been shown to be the cytoplasmic adaptor protein essential for IL-1 and IL-18 signaling (Muzio et al., 1997; Wesche et al., 1997a; Adachi et al., 1998). Increased resistance is also observed in mice lacking IL-1β-converting enzyme (ICE), which is essential for production of mature and active forms of both IL-1β and IL-18 (Li et al., 1995; Ghayur et al., 1997; Gu et al., 1997). Therefore, we challenged mice lacking type I IL-1R and/or IL-18 with high doses of LPS. These mice did not show any increased resistance to LPS-induced endotoxin shock, demonstrating that the hyporesponsiveness to LPS in MyD88 mice is not due to a lack of IL-1α/β and/or IL-18 action (Figure 1A). We measured serum concentrations of inflammatory cytokines after LPS challenge in MyD88 KO mice. Increases in serum IL-6, tumor necrosis factor-α (TNFα), and IL-1β levels were not observed in MyD88 KO mice (Figure 1B).

Normal Expression of CD14, TLR2, TLR4, and SLPI in MyD88 KO Mice

As macrophages are major target cells for LPS, we examined expression of molecules that are critical for LPS signaling in macrophages. Expression of CD14, one of the well-known receptors for LPS, on the cell surface of macrophages from MyD88 KO mice was analyzed by flow cytometry. CD14 expression on peritoneal macrophages was not altered between wild-type and MyD88 KO mice (Figure 2A). Recent reports have demonstrated that human TLR2 is a surface molecule that binds to

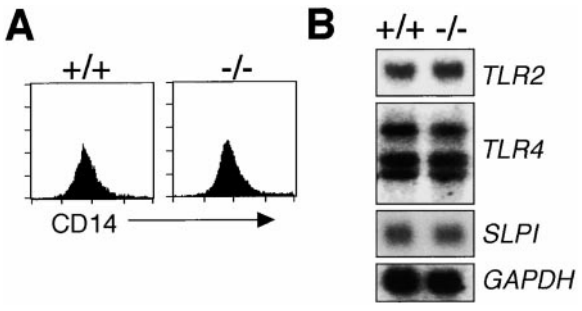

Figure 2. Expression of CD14, TLR2, TLR4, and SLPI in MyD88 KO Macrophages

(A) Peritoneal exudate cells were doubly stained with PE-conjugated anti-CD14 antibody and FITC-conjugated anti-Mac1 antibody. Stained cells were analyzed on FACS Calibur using Cell Quest software. Expression of CD14 on Mac1-positive cells was shown.

(B) Peritoneal macrophages were isolated 4 days after intraperitoneal thioglycollate injection. Total RNA was extracted and subjected to Northern blot analysis using cDNA probes for TLR2, TLR4, and SLPI. The same membrane was rehybridized with a GAPDH probe.

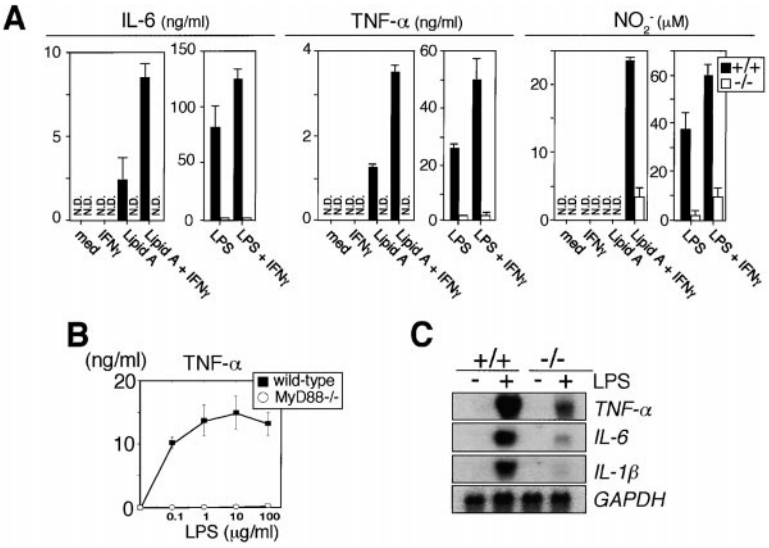

Figure 3. Lack of LPS Responsiveness in MyD88 KO Macrophages

(A) Peritoneal macrophages were isolated and cultured with 2.0 μg/ml LPS from *E. coli* serotype O55:B5 or 1.0 μg/ml synthetic *E. coli*–type lipid A (compound 506) in the presence or absence of 30 U/ml IFNγ for 24 hr. Concentrations of TNFα, IL-6, and NO_2^- in the culture supernatants were measured by ELISA.

(B) Peritoneal macrophages from wild-type and MyD88 KO mice were cultured with the indicated concentrations of O55:B5 LPS for 24 hr. Concentrations of TNFα in the culture supernatants were measured.

(C) Peritoneal macrophages from wild-type and MyD88 KO mice were stimulated with 2.0 μg/ml O55:B5 LPS for 1.5 hr. Total RNA was extracted and subjected to Northern blot analysis using cDNA probes for TNFα, IL-6, and IL-1β. The same membrane was rehybridized with a GAPDH probe.

LPS (Kirschning et al., 1998; Yang et al., 1998) and that TLR4 is the gene product of Lps locus, the defect of which results in hyporesponsiveness to LPS in C3H/HeJ mice (Poltorak et al., 1998; Hoshino et al., 1999; Qureshi et al., 1999). We next analyzed expression of TLR2 and TLR4 in macrophages by Northern blot analysis. Nontreated peritoneal macrophages from both wild-type and MyD88 KO mice expressed TLR2 and TLR4 mRNAs to similar extents (Figure 2B). We also analyzed mRNA expression of SLPI, a secretory protein that inhibits the responsiveness to LPS (Jin et al., 1997). Both wild-type and MyD88 KO macrophages displayed almost the same level expression of SLPI mRNA (Figure 2B).

MyD88 KO Macrophages Failed to Produce Any Inflammatory Cytokines or Mediator in Response to LPS

Next, we examined the LPS responsiveness of MyD88 KO macrophages using *Escherichia coli* LPS (O55:B5) and synthetic *E. coli*–type lipid A (compound 506), a biologically active moiety of LPS. Peritoneal macrophages were cultured with LPS (O55:B5) or compound 506 in the presence or absence of interferon-γ (IFNγ) for 24 hr, and productions of TNFα, IL-6, and nitric oxide (NO_2^-) were measured (Figure 3A). Wild-type macrophages produced IL-6, TNFα in response to compound 506 and LPS, and showed an enhanced production of these cytokines when the cells were cocultured with IFNγ. In particular, stimulation with LPS resulted in secretion of a high amounts of cytokines when compared with compound 506. On the other hand, macrophages from MyD88 KO mice did not secrete any detectable levels of IL-6 and TNFα in response to LPS or IFNγ plus LPS. Production of NO_2^- from macrophages was dramatically induced in response to LPS plus IFNγ in wild-type mice. In contrast, NO_2^- production was only slightly increased in MyD88 KO macrophages. We next cultured peritoneal macrophages in the presence of various concentrations of LPS, and production of TNFα in the culture supernatant was measured. Wild-type macrophages produced an increased level of TNFα in response to LPS in a dose-dependent manner. In contrast, MyD88 KO macrophages did not produce TNFα at all, even when these cells were cultured with a high concentration of LPS (Figure 3B). LPS-induced mRNA expression of these cytokines was analyzed by Northern blot. In wild-type mice, treatment with LPS induced the significant increase in mRNA expression for TNFα, IL-6, and IL-1β. However, a marginal or no induction of mRNA for TNFα, IL-6, and IL-1β was observed in MyD88 KO macrophages (Figure 3C). These data demonstrate that MyD88 KO macrophages displayed almost complete loss of LPS responsiveness.

Unresponsiveness to LPS-Induced Expression of MHC Class II and Proliferation of B Cells in MyD88 KO Mice

B cells are also shown to respond to LPS. We next analyzed LPS responsiveness of B cells. Flow cytometric analysis demonstrated that B cell population and their surface expression of B220, IgM, and IgD were not altered in MyD88 KO mice, indicating that B cell development was not affected in MyD88 KO mice (data not shown). Splenic B cells were purified and cultured in the presence of various concentrations of LPS (O55:B5). Wild-type B cells showed an increased proliferative response to LPS in a dose-dependent manner. However, MyD88 KO B cells did not proliferate in response to LPS even when cultured in high concentrations (Figure 4A). We further analyzed surface expression of MHC class II in response to LPS on B cells. Wild-type B cells showed an increased expression of MHC class II in response to LPS. However, LPS-induced augmentation of MHC class II expression on the cell surface of MyD88 KO B cells was almost completely impaired (Figure 4B). Both wild-type and MyD88 KO B cells expressed almost the same level of MHC class II in response to IL-4. Thus, B cells of MyD88 KO mice are specifically unresponsive to LPS.

We further examined LPS responsiveness of embryonic fibroblast (EF) cells. Wild-type EF cells produced a high level of IL-6 in response to LPS (O55:B5). However, EF cells from MyD88 KO mice did not produce any detectable levels of IL-6 in response to LPS, in contrast

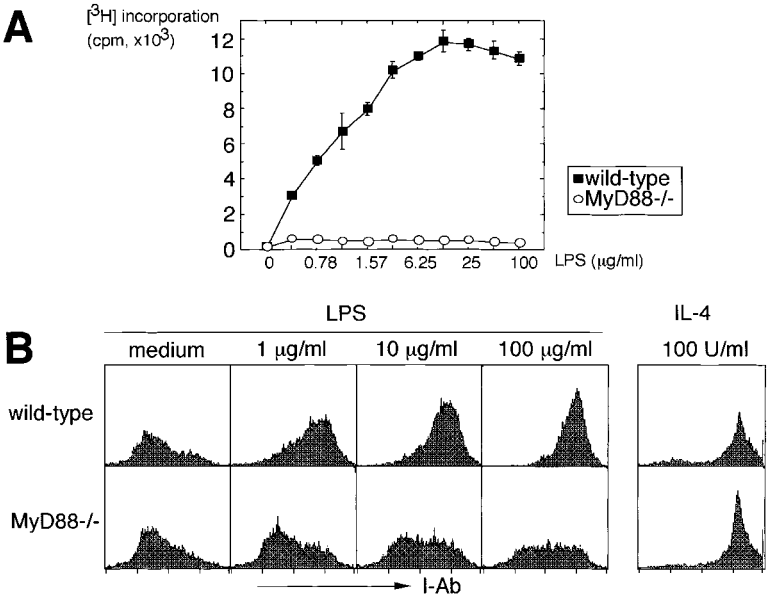

Figure 4. Lack of LPS Responsiveness in MyD88 KO B Cells

(A) Splenic B cells were negatively enriched from wild-type and MyD88 KO mice by magnetic cell sorting (MACS; Miltenyi Biotec) using anti-Thy1 microbeads, and purified cells were cultured with the indicated concentrations of O55:B5 LPS for 48 hr. [^{3}H]thymidine (1.0 μCi) was pulsed for the last 6 hr. [^{3}H] incorporation was measured by a scintillation counter (Packard).

(B) Splenic B cells were purified and cultured with the indicated concentrations of O55:B5 LPS or 100 U/ml IL-4. At the 48 hr culture period, cells were harvested and stained with biotin-conjugated anti-I-Ab antibody followed by streptavidin-PE. Stained cells were analyzed on FACS Calibur using Cell Quest software.

to the normal production of IL-6 in response to TNFα (data not shown). Taken together, these data show that macrophages, B cells, and fibroblasts from MyD88 KO are unresponsive to LPS, demonstrating that MyD88 is essential for LPS response.

Stimulation of Lipid A Induced the Activation of IRAK in Wild-Type Macrophages but Not in MyD88 KO Macrophages

To assess the molecular mechanism of unresponsiveness to LPS in MyD88 KO mice, we examined whether or not LPS-mediated signaling cascades were impaired. In this experiment, we used synthetic *E. coli*-type lipid A (compound 506) in order to rule out contamination of proteins. IRAK was originally identified as a protein kinase that associates with IL-R complex after IL-1 stimulation (Cao et al., 1996a; Huang et al., 1997; Wesche et al., 1997b). MyD88 is shown to associate with IRAK through the death domain and functions as an adoptor between IL-1R and IRAK (Muzio et al., 1997; Wesche et al., 1997a). Therefore, we first examined the activation of IRAK after LPS stimulation. Peritoneal macrophages from wild-type and MyD88 KO mice were treated with compound 506 and then lysed and immunoprecipitated with anti-IRAK1 antibody. IRAK activity was measured by in vitro kinase assay (Kojima et al, 1998). As shown in Figure 5, autophosphorylation of IRAK is detected at 10 min after compound 506 stimulation in wild-type macrophages. In contrast, IRAK activation in response to compound 506 was completely abolished in MyD88 KO macrophages, demonstrating that MyD88 is a critical molecule for the activation of IRAK in response to lipid A.

Delayed Activation Profile of the MAP Kinase Family and NF-κB in Response to Lipid A in MyD88 KO Macrophages

We next analyzed lipid A–induced tyrosine phosphorylation of the cytoplasmic proteins by Western blot analysis with anti-phospho-tyrosine antibody. As shown in Figure 6A, several cytoplasmic proteins were tyrosine phosphorylated in wild-type macrophages within 10 to 20 min following the addition of compound 506. On the other hand, tyrosine phosphorylation in MyD88 KO macrophages was detected at 20 min but not at 10 min. Furthermore, we used Polymixyin B, a polycationic antibiotic that inhibits LPS-induced activation of cells by binding the lipid A region, to rule out the possibility of cell activation by contaminated material(s) other than lipid A. Treatment of macrophages from wild-type and MyD88 KO mice with 100 U/ml Polymixyin B prior to stimulation with compound 506 resulted in a complete inhibition of the induction of protein tyrosine phosphorylation, confirming that compound 506 induced-protein tyrosine phosphorylation in MyD88 KO macrophages is not due to contaminated material(s) (Figure 6B). We also analyzed the activation of the MAP kinase family members including c-Jun N-terminal kinase (JNK), ERK1,2, and p38. We measured the activity of JNK by in vitro kinase assay using GST-c-Jun as the substrate. As shown in Figure 6C, JNK was activated in response to compound 506 in wild-type macrophages. On the other

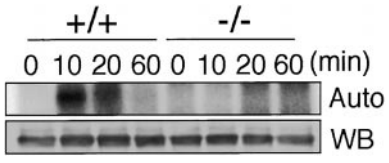

Figure 5. Lipid A Treatment Induces the Activation of IRAK in Wild-Type but Not in MyD88 KO Macrophages

Peritoneal macrophages were stimulated with 2.0 μg/ml synthetic lipid A (compound 506) for 10, 20, or 60 min. The cell lysates were prepared and immunoprecipitated with anti-IRAK antibody. The kinase activity of IRAK was measured by in vitro kinase assay (upper panel). The same lysates were blotted with anti-IRAK antibody to monitor the expression (lower panel). Similar results were obtained from three independent experiments. Auto, autophosphorylation.

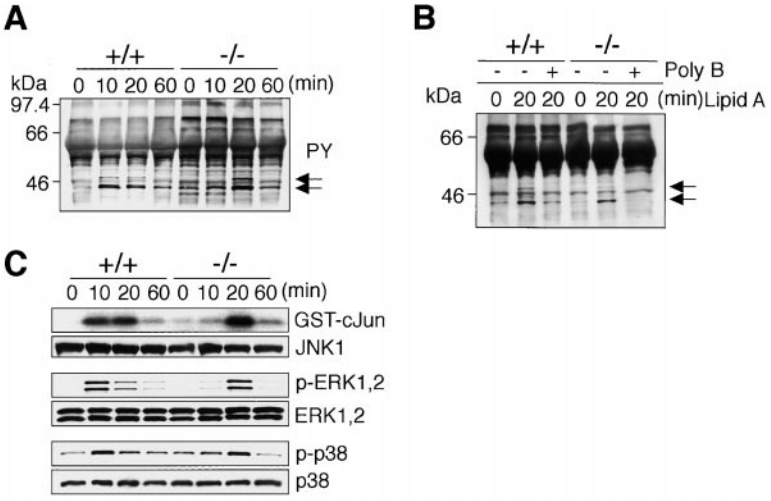

Figure 6. Delayed Activation of the MAP Kinase Family after Lipid A Stimulation in MyD88 KO Macrophages

(A) Peritoneal macrophages stimulated with 2.0 μg/ml compound 506 for 10, 20, or 60 min were lysed and blotted with anti-phosphotyrosine 4G10 antibody. The bound antibody was visualized with an enhanced chemiluminescence system. Inducible tyrosine-phosphorylated proteins are indicated by arrows. Similar results were obtained from three independent experiments.

(B) Peritoneal macrophages were incubated wtih 100 U/ml Polymixyin B for 20 min and then stimulated with 2.0 μg/ml compound 506 for 20 min. The whole cell lysates were blotted with anti-phosphotyrosine 4G10 antibody.

(C) Peritoneal macrophages were stimulated with 2.0 μg/ml compound 506 for the indicated periods. The cell lysates were immunoprecipitated with anti-JNK1 antibody. The JNK activity in the immunoprecipitates was measured by in vitro kinase assay using GST-cJun-fusion protein as a substrate (GST-cJun). The same lysates were blotted with anti-JNK1 antibody to monitor the expression (JNK1). Activation of ERK1,2 or p38 was measured by Western blot analysis with antibodies specific for tyrosine-phosphorylated ERK1,2 (p-ERK1,2) or p38 (p-p38), respectively. The same lysates were blotted with anti-ERK1,2 (ERK1,2) or p38 (p38) antibody to monitor the expression. Similar results were obtained from three independent experiments.

hand, MyD88 KO macrophages showed the delayed activation of JNK. Activation of ERK1,2 and p38 in response to compound 506 was measured by Western blotting with antibodies specific for tyrosine-phosphorylated ERK1,2 and p38, respectively. As shown in Figure 6C, tyrosine phosphorylation of ERK1,2 and p38 in MyD88 KO macrophages was also delayed as compared with wild-type macrophages.

We next analyzed lipid A–induced NF-κB activation by electrophoretic mobility shift assay. As shown in Figure 7A, NF-κB DNA binding activity was detected in both the nuclear extracts from wild-type and MyD88 KO macrophages at 60 min after compound 506 stimulation, and the NF-κB DNA complex was supershifted with antibody to p50. The nuclear extracts from compound 506–stimulated MyD88 KO macrophages showed almost comparable NF-κB DNA binding activity and complex composition with wild-type macrophages (Figure 7A). We further analyzed lipid A–induced activation of NF-κB at different time points. In wild-type macrophages, compound 506–induced NF-κB DNA binding activity was detected at 10 min and sustained up to 60 min (Figure 7B). On the other hand, NF-κB activation was not detected at 10 min but at 20 min in MyD88 KO macrophages. Thus, NF-κB activation after lipid A stimulation was also delayed, albeit similarly induced, in MyD88 KO macrophages. Next, we tested whether treatment with a low concentration of lipid A also activates NF-κB in MyD88 KO macrophages. As shown in Figure 7C, NF-κB was also activated in cells stimulated with 1.0 ng/ml compound 506 from both wild-type and MyD88 KO mice as measured by electrophoretic mobility shift assay, indicating that activation of NF-κB obtained from MyD88 KO macrophages is not due to a

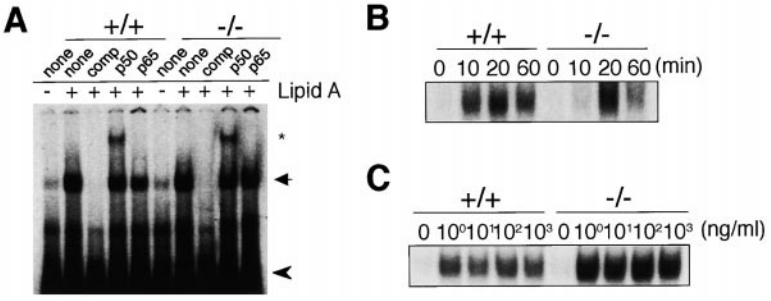

Figure 7. Delayed Activation of NF-κB after Lipid A Stimulation in MyD88 KO Macrophages

(A) Peritoneal macrophages from wild-type or MyD88 KO mice were stimulated with 2.0 μg/ml compound 506 for 60 min. Nuclear extracts were then prepared and incubated with a specific probe containing NF-κB binding sites, and NF-κB activity was determined by electrophoretic mobility shift assay. Specificity was determined by adding 50-fold molar excess of specific competitor (comp) or 1.0 μg of specific antibodies to p50 (p50) and p65 (p65). Inducible NF-κB complex was indicated by the arrow. An asterisk indicates the supershift bands. The arrowhead indicates the free probe. Similar results were obtained from three independent experiments.

(B) Peritoneal macrophages from wild-type or MyD88 KO mice were stimulated with 2.0 μg/ml compound 506 for the indicated periods. Nuclear extracts were then prepared, and NF-κB activity was determined by electrophoretic mobility shift assay. Similar results were obtained from three independent experiments.

(C) Peritoneal macrophages from wild-type or MyD88 KO mice were stimulated with the indicated concentration of compound 506 for 60 min. Nuclear extracts were then prepared, and NF-κB activity was determined by electrophoretic mobility shift assay. Similar results were obtained from three independent experiments.

result from the effect that stimulation with a high concentration of lipid A may cause activation of more than one signaling pathway. Furthermore, stimulation with 055:B5 LPS also significantly induced the activation of NF-κB and MAP kinases in macrophages from both wild-type and MyD88 KO mice as in the case with lipid A stimulation (data not shown).

The present study demonstrates that MyD88 KO mice lack responsiveness to LPS. MyD88 KO mice are resistant to endotoxin shock in response to high doses of LPS. Serum levels of cytokines such as IL-6, TNFα, and IL-1β were not increased in MyD88 KO mice after LPS injection. Macrophages from MyD88 KO mice did not produce any detectable levels of IL-6 and TNFα and produced a minor amount of NO_2^- in response to LPS plus IFNγ. Furthermore, B cells were also unresponsive to LPS, demonstrating that MyD88 is a critical molecule for LPS response.

TLR2 and TLR4 have been suggested to be the functional receptors for LPS. Since the cytoplasmic region of the TLR family member shares homology to that of the IL-1R family, TLRs are also expected to use the same molecules with IL-1R for its signaling. TLR4 is reported to activate NF-κB through the MyD88- and TRAF6-dependent pathway (Muzio et al., 1998). Therefore, it is considered that MyD88 is a critical molecule for both LPS and IL-1R signalings. In fact, IRAK is activated in response to LPS in wild-type but not MyD88 KO mice, indicating that IRAK is an LPS-responsive kinase that acts downstream to MyD88. However, both MAP kinases and NF-κB were activated in MyD88 KO mice, although the activation of these molecules was delayed when compared with wild-type mice. This finding markedly contrasts with the case of IL-1 and IL-18 signaling in MyD88 KO mice, in which both NF-κB and JNK activation was completely blocked (Adachi et al., 1998).

The responsiveness to LPS in MyD88 KO mice is quite similar to that in C3H/HeJ mice (Morrison and Ryan, 1979), in which small responses are observed by stimulation with high concentrations of LPS or after a long exposure to LPS. In fact, macrophages from C3H/HeJ mice display the same electrophoretic mobilites and content of heterodimers of NF-κB subunits in the NF-κB-containing DNA-protein complexes after LPS stimulation as responder macrophages (Ding et al., 1995). Furthermore, the phenotype of MyD88 KO mice seems to be quite similar to that of LPS tolerant mice that exhibit a profound refractoriness to LPS after repeated stimulation, although LPS signal can be transmitted from the cell surface to the interior (Nakano et al., 1993; Takasuka et al., 1995; Ziegler-Heitbrock, 1995; Bohuslav et al., 1998). The LPS signaling cascade may be composed of MyD88-dependent and -independent pathways, both of which lead to the activation of the MAP kinase family and NF-κB. The MyD88-dependent pathway could activate an unidentified but unique signaling molecule that is essential for LPS response. Although the precise mechanisms by which MyD88 deficiency results in LPS unresponsiveness are not well understood, the present study clearly demonstrates that signaling via MyD88 is essential for LPS response.

Experimental Procedures

Reagents and Animals

LPS from *E. coli* serotype O55:B5 (phenol extracted and then chromatographically purified by gel filtration) was purchased from Sigma. LPS was solubilized in distilled water by sonication. *E. coli*–type synthetic lipid A (compound 506) was purchased from Daiichi Pure Chemicals, Tokyo, and solubilized in distilled water containing 0.025% of triethylamine. Polymixyn B was purchased from Sigma. IFNγ and IL-4 were purchased from Genzyme.

Peritoneal macrophages and B cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). MyD88 KO mice were generated and maintained as described previously (Adachi et al., 1998). IL-18 KO mice were as described previously (Takeda et al., 1998). Type I receptors for IL-1 KO mice were kindly provided from Immunex (Glaccum et al., 1997). IL-18 KO mice were crossed with IL-1R KO mice to generate mice heterozygous for both the IL-1R and IL-18 genes. Offsprings from these intercrosses were used for the experiments.

ELISA

Production of IL-1β, IL-6, and TNFα from peritoneal macrophages was measured by ELISA according to the manufacturer's instructions (Genzyme). Production of NO_2^- was measured by NO_2/NO_3 Assay Kit-C (DOJINDO).

Northern Blot Analysis

Total RNA was extracted using the TRIzol reagent (Gibco). Ten micrograms of total RNA was electrophoresed, transferred to a nylon membrane, and hybridized with cDNA probes as described previously (Adachi et al., 1998). cDNA probes specific for TNFα, IL-6, IL-1β, TLR2, TLR4, and SLPI were obtained by PCR with a set of specific primers from a mouse peritoneal macrophage cDNA library. The primer sequences are available upon request.

In Vitro Kinase Assay

Peritoneal macrophages stimulated with compound 506 were lysed and immunoprecipitated with anti-IRAK antibody, and IRAK activity was measured by in vitro kinase assay as described previously (Kojima et al., 1998). Anti-IRAK antibody was kindly provided by Hayashibara Biochemical Laboratories.

JNK activity in the immunoprecipitates with anti-JNK1 antibody was examined by in vitro kinase assay using GST-c-Jun fusion protein as the substrate as described previously (Adachi et al., 1998).

Western Blot Analysis

Peritoneal macrophages were treated with compound 506 for the indicated periods. The cells were then 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 blotted with antibody to JNK1 (Santa Cruz), ERK (Santa Cruz), p38 (Santa Cruz), phosphorylated-ERK (New England Biolabs), phosphorylated-p38 (New England Biolabs), IRAK (Transduction Laboratories), or phospho-tyrosine 4G10 (Upstate Biotechnology), and visualized using the enhanced chemiluminescence system (Dupont).

Electrophoretic Mobility Shift Assay

The nuclear extracts of peritoneal macrophages (5×10^6) were purified after compound 506 stimulation. The extract was incubated with a specific probe for NF-κB DNA binding site, electrophoresed, and visualized by autoradiography as described previously (Adachi et al., 1998). Antibodies to p50 and p65 were purchased from Santa Cruz.

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