Dual Receptors and Distinct Pathways Mediate Interleukin-1 Receptor-associated Kinase Degradation in Response to Lipopolysaccharide

IN Volvement of CD14/TLR4, CR3, and Phosphatidylinositol 3-Kinase*

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Lipopolysaccharide (LPS) signaling leading to nuclear factor-κB activation in mononuclear phagocytes involves interleukin-1 receptor-associated kinase (IRAK), which is rapidly activated after exposure to agonist. Although it is known that IRAK also undergoes rapid inactivation/degradation in response to LPS, providing negative feedback leading to LPS tolerance, mechanisms governing IRAK degradation are not fully understood. In the present study, examination of LPS signaling showed that IRAK degradation was bimodal and involved dual receptors and distinct pathways. Rapid degradation of IRAK, occurring within 30 min of exposure to agonist, was shown to signal through CD14/TLR4, and was regulated by phosphatidylinositol 3-kinase. A second delayed wave of IRAK degradation occurred 2 h after exposure to LPS and was mediated by CR3 independently of phosphatidylinositol 3-kinase. Thus, multiple independent mechanisms have evolved to regulate IRAK degradation, likely reflecting the importance of limiting cellular responses to LPS. Recognition of a CR3-dependent, CD14/TLR4-independent pathway leading to IRAK degradation has implications for understanding modulation of LPS responses by cells with important immunoregulatory function such as dendritic cells that are CD14+.

Bacterial lipopolysaccharide (LPS) stimulation of normal human macrophages leads to production of a wide range of inflammatory mediators, most notably interleukin-1β and tumor necrosis factor-α as well as many other bioactive molecules (1). Finite amounts of these mediators are clearly essential for orchestrating diverse effector mechanisms of innate immunity; however, unrestrained production associated with sepsis results in severe systemic inflammatory complications (2). To date, given these severe and often fatal consequences, a reasonable host survival strategy would be to invoke mechanisms to limit the response to LPS. Indeed, examination of neutrophils and monocytes isolated from patients with sepsis has shown that these cells are relatively unresponsive to LPS challenge in vitro and no longer produce inflammatory cytokines (3). This phenomenon has been referred to as endotoxin tolerance, and it has been possible to develop in vitro models with similar properties which are amenable to study. Thus, when macrophages are cultured in vitro in the presence of LPS for prolonged periods they become refractory to subsequent challenge with LPS and no longer express inflammatory cytokines (3).

The human promonocytic cell line THP-1 has proven to be useful for studying LPS tolerance. These cells exhibit nearly all of the properties observed when studying monocytes and neutrophils from patients with sepsis (4). For example, both human leukocytes obtained from patients with sepsis and THP-1 cells exposed to LPS in vitro show attenuated innate immune activation, as reflected by the markedly reduced levels of steady-state mRNAs for proinflammatory mediators (4).

Although there is extensive knowledge regarding functional changes in monocytes induced by LPS, comparatively little is known about the regulation of LPS tolerance. Genetic and other evidence has established that LPS signals predominantly through TLR4 (5, 6) and utilizes signaling elements in common with the interleukin-1 receptor including MyD88, IRAK, and TRAF6 to elicit cellular responses (7). TIRAP, also known as MAL, an adaptor protein in the TLR signaling pathway, has also been identified and shown to function downstream of TLR4 (8, 9).

To function as an LPS receptor, TLR4 must interact with a secreted protein, MD-2 (10–12). Site-directed mutagenesis has shown that these cells are relatively unresponsive to LPS challenge in vitro and no longer produce inflammatory cytokines (3). For example, both human leukocytes obtained from patients with sepsis and THP-1 cells exposed to LPS in vitro show attenuated innate immune activation, as reflected by the markedly reduced levels of steady-state mRNAs for proinflammatory mediators (4).
Receptors and Mechanisms of IRAK Degradation

The hypothesis that PI 3-kinase regulates LPS-induced IRAK degradation. The results show that IRAK degradation in response to LPS is in fact bimodal with both rapid and delayed kinetics induced by distinct receptors, respectively, CD14 and CR3. Moreover, only CD14 seems to require TLR4 as a coreceptor to induce IRAK degradation. Consistent with the model proposed, the rapid phase of IRAK degradation is PI 3-kinase-dependent, whereas the delayed phase is not.

**EXPERIMENTAL PROCEDURES**

**Reagents and Chemicals—**RPMI 1640 and penicillin/streptomycin were purchased from Stem Cell Technologies (Vancouver, BC). Wortmannin, t-α-phosphatidylinositol, phenethylsulfonyl fluoride, leupeptin, pepstatin A, and apronin were from Sigma. LY294002 and G418 were purchased from Calbiochem. Protein A-agarose and electrorephoresis reagents were from Bio-Rad Laboratories. γ-32P[ATP] was from PerkinElmer Life Sciences.

**Antibodies—**Antihuman CR3 mAbs (clone LM1/20 and M1/70) were kindly provided by Dr. W. C. Van Voorhis (University of Washington, Seattle). Clones 63D3 and 28C5 were a gift from Dr. P. S. Tobias (The Scripps Research Institute, La Jolla, CA). Anti-CD14 mouse mAb (HTA125) and its purified mouse IgG2a control were purchased from Santa Cruz Biotechnology. Anti-PI 3-kinase mAb (clone UB93-3) and rabbit anti-IRAK were from Upstate Biotechnology (Lake Placid, NY). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was obtained from Sigma.

**Cell Lines—**The monocytic cell lines THP-1wt (THP-1 cells stably expressing glycosylphosphatidylinositol-anchored CD14) and THP-1ev (THP-1 cells transfected with vector alone) were kindly provided by Dr. R. Ulevitch (The Scripps Research Institute). THP-1 cells were cultured in RPMI supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml G418 and maintained at a concentration below 5 × 10^6/ml.

**Cell Surface Phenotyping—**To measure the expression of surface molecules, cells were incubated with specific mouse mAb or irrelevant isotype-matched IgG (10 μg/ml) for 30 min, washed twice, and labeled with fluorescein isothiocyanate-conjugated Fab′1, goat anti-mouse IgG for 30 min. Cells were then washed twice and fixed in 2% paraformaldehyde in the same binding buffer. All staining and washing procedures were performed at 4 °C in Hanks’ balanced salt solution containing 0.1% Na_N_3 and 1% fetal calf serum. Cell fluorescence was analyzed using a FACS Calibur flow cytometer (BD Biosciences). Relative fluorescence intensities of 5,000–10,000 cells were recorded as single parameter histograms (log scale, 1,024 channels, 4 log decades), and the mean fluorescence intensity (MFI) was calculated for each histogram. Results are expressed as MFI indices, which correspond to MFI of cells + specific Ab/MFI of cells + irrelevant isotype-matched IgG.

**Detection of IRAK-1 Degradation—**THP-1 cells were washed with serum-free RPMI medium resuspended in culture medium at a final concentration of 5 × 10^5 cells/ml and stimulated with Escherichia coli LPS (serotype 0111:B4, Sigma). Cells were then washed with Hank’s balanced salt solution, and pellets were solubilized in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1 mM EDTA, 137 mM NaCl, 1 mM phenethylsulfonyl fluoride, 2 mM sodium vanadate, 2 mM NaF, pepstatin, leupeptin, and aprotonin). Cell debris was pelleted by centrifugation for 20 min at 10,000 × g, and protein concentration in the supernatant was determined using a Bradford protein-assay kit (Bio-Rad). Equal amounts of protein samples (~50 μg) were mixed with Laemmli buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were probed with anti-IRAK or anti-actin antibodies and developed by ECL.

**In Vitro PI 3-Kinase Assay—**Cell lysates for analysis of PI 3-kinase were prepared in 20 mM Tris, pH 8.0, 1% Triton X-100, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 100 μM microcystin, 1 mM phenethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin. Aliquots of lysates adjusted for protein concentration (500 μg of protein) were incubated for 4 h at 4 °C with UB93-3 mAb (anti-PI 3-kinase), and immune complexes were adsorbed onto protein A-agarose for 1 h. The complexes were washed twice with lysis buffer and three times with 10 mM Tris-HCl, pH 7.4. To ensure that PI 3-kinase levels remained equivalent at the end of the immunoprecipitation, 10% from each treatment sample was collected during the last wash in a separate tube and analyzed by SDS-PAGE and immunoblotting with Abs to the p85 PI 3-kinase...
two independent experiments that yielded similar results. The results shown correspond to one of the TLC plate followed by liquid scintillation counting.

**RESULTS**

Analysis of CD14 and CR3 Surface Expression by THP-1rw and THP-1wt Cells—Surface expression of CD14 and CR3 molecules was analyzed by immunofluorescence and fluorescence-activated cell sorter analysis. The representative record shown in Fig. 1 demonstrates that cells transfected with CD14 (THP-1wt) and control cells transfected with vector alone (THP-1rsv) expressed negligible numbers of CD14 molecules on the cell surface (MFI, 1.5), THP-1wt cells expressed ~80 times more CD14 (MFI, 120).

**Rapid Degradation of IRAK Requires Expression of CD14**—Because both CD14 and CR3 have been implicated in the LPS-induced signaling (25, 31, 32) we examined whether either may be involved in IRAK degradation. To address this, THP-1rsv (CR3+/CD14−) and THP-1wt (CR3+/CD14+) cells were incubated with a range of concentrations of LPS for 30 min or 2 h. Both THP-1rsv and THP-1wt showed LPS dose-dependent reductions in IRAK levels. As seen in Fig. 2, THP-1wt showed substantial degradation of IRAK after only a 30-min exposure to 100 ng/ml LPS, and this was even more marked with LPS at 500 ng/ml. In contrast, THP-1rsv cells were completely resistant to LPS at these concentrations with respect to changes in IRAK levels during a 30-min incubation. In fact, reductions in IRAK levels in THP-1rsv cells were only apparent after a 2-h incubation with LPS in the range of 0.5–1.0 μg/ml.

A more detailed time course analysis confirmed that the kinetics of IRAK degradation were clearly distinct for these two cell types. Thus, as shown in Fig. 3, when both THP-1wt and THP-1rsv cells were incubated with 500 ng/ml LPS, THP-1wt cells showed evidence of IRAK degradation within 15 min of exposure, and the level of IRAK was less than 10% of control by 30 min after the addition of LPS. In contrast, no significant change was observed for THP-1rsv cells within this time frame. In fact, significant reductions in IRAK levels were only apparent in THP-1rsv cells beginning after 1 h of the LPS addition and were maximal after 2 h, at which time IRAK content in THP-1wt cells was undetectable. These findings show that both rapid and delayed phases of IRAK degradation occur and that the former requires expression of CD14.

**PI 3-Kinase Regulates the Rapid but Not the Late Phase of IRAK Degradation in Response to LPS**—Based upon the findings that LPS activates PKC-ζ in a PI 3-kinase dependent manner (39) and that PKC-ζ may be involved in IRAK degradation (40), we examined the hypothesis that PI 3-kinase regulates LPS-induced IRAK degradation. THP-1wt cells were incubated for 20 min with either of two unrelated PI 3-kinase inhibitors, LY294002 (20 μM) or wortmannin (50 nM), prior to the addition of LPS for either 30 min or 2 h. The levels of IRAK protein were then analyzed by Western blotting. As expected, LPS treatment resulted in a marked reduction in IRAK levels both at 30 min and 2 h after the addition of agonist to THP-1wt cells (Fig. 4). Of interest, pretreatment of cells with either PI 3-kinase inhibitor completely abrogated IRAK degradation brought about by 30 min of exposure to LPS. Strikingly, despite the elimination of this rapid phase of IRAK degradation, by 2 h after LPS exposure IRAK levels were less than 10% of those seen in non-LPS-treated control cells, despite the continuous presence of either wortmannin or LY294002. Thus, PI 3-kinase appears to be required for rapid but not late phase IRAK degradation.

**CD14 Is Involved in the Rapid but Not in the Late Phase of IRAK Degradation in Response to LPS**—Although the data thus far were consistent with CD14 being involved in IRAK degradation, it was not clear whether this LPS receptor was involved in both early and late phases. Given that low levels of CD14 molecules are detectable on THP-1rsv cells, it was not possible to exclude their involvement fully.
CR3 Is Involved in Inducing Late Phase IRAK Degradation—Like CD14, CR3 and CR4 have been reported to bind LPS leading to signal transmission (25, 46, 47), and this may involve some of the same signaling elements downstream of CD14. To examine whether CR3 may function as an LPS receptor responsible for inducing delayed, PI 3-kinase-independent IRAK degradation, THP-1rsv cells were preincubated for 30 min with two different anti-CD14 mAbs (clone 63D3 or 28C5, final concentration 50 μg/ml) or medium alone for 30 min at room temperature then treated with 500 ng/ml LPS for 30 min (A) or 2 h (B). Cells were then washed, lysed, and equal amounts of proteins were analyzed by SDS-PAGE and Western blotting with anti-IRAK-1 antibodies. The results shown are one of three experiments with similar results.

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under these conditions, it is not surprising that a variety of control mechanisms have evolved to exert a negative feedback regulation of cellular responses to LPS. These include the concomitant production of anti-inflammatory cytokines or other proteins capable of binding LPS and inhibiting its actions such as bacterial permeability-increasing protein (50–52). An additional level at which this type of control may be exerted involves attenuation of LPS signal transmission, and recent findings suggest that one such key control point may be IRAK. It has been known for many years that macrophages and other phagocytic cells incubated with LPS become unresponsive or tolerant to second challenge with LPS (3). Recently, it has been shown that in response to prolonged LPS exposure, cellular levels of IRAK protein and correspondingly IRAK activity are markedly diminished, and this correlates tightly with LPS tolerance (18). In the latter study, which examined normal THP-1 cells, which express little or no CD14, evidence for changes in IRAK abundance and activity was not evident until at least 1.5 h after the addition of LPS. However, several lines of evidence suggest that more rapid degradation of IRAK occurs under these conditions, it is not surprising that a variety of control mechanisms have evolved to exert a negative feedback regulation of cellular responses to LPS. These include the concomitant production of anti-inflammatory cytokines or other proteins capable of binding LPS and inhibiting its actions such as bacterial permeability-increasing protein (50–52). An additional level at which this type of control may be exerted involves attenuation of LPS signal transmission, and recent findings suggest that one such key control point may be IRAK. It has been known for many years that macrophages and other phagocytic cells incubated with LPS become unresponsive or tolerant to second challenge with LPS (3). Recently, it has been shown that in response to prolonged LPS exposure, cellular levels of IRAK protein and correspondingly IRAK activity are markedly diminished, and this correlates tightly with LPS tolerance (18). In the latter study, which examined normal THP-1 cells, which express little or no CD14, evidence for changes in IRAK abundance and activity was not evident until at least 1.5 h after the addition of LPS. However, several lines of evidence suggest that more rapid degradation of IRAK in response to LPS is likely to occur in cells that are CD14⁺. For example, in interleukin-1-treated MRC-5 human lung fibroblasts, as IRAK became polyphosphorylated it was rapidly degraded by proteasomes at a rate comparable with that of the degradation of phosphorylated IκB-α (53). Second, in THP-1 cells as well as in the murine macrophage cell lines HeNC2 and GG2EE, IRAK was shown to undergo degradation within 15 min after treatment with Gram-negative flagellin (54). Third, the proteasome acts constitutively and does not require de novo protein synthesis in response to stimuli (55). Fourth, because CD14 is the primary receptor linked to the dominant LPS signaling pathway through TLR4, it is likely that optimal conditions for IRAK degradation include expression of CD14. In fact, the prediction that rapid IRAK degradation is likely to be induced by CD14 proved to be correct. Thus, detailed kinetic analysis comparing both CD14⁻ and CD14⁺ cells (Figs. 2 and 3) indicated that the expression of CD14 confers a phenotype of rapid IRAK degradation in response to LPS, a property not shared by cells expressing low or undetectable levels of CD14. Whereas CD14⁻ cells were also capable of degrading IRAK, this occurred with distinctly delayed kinetics, providing evidence for bimodal regulation of IRAK expression.

The discovery that IRAK degradation was in fact bimodal suggested the possibility that it might be regulated by multiple mechanisms. This inference was confirmed with the use of blocking antibodies that established that only the rapid phase of IRAK degradation was TLR4- and CD14-dependent (Fig. 5). To address how the delayed phase was regulated, we considered other putative LPS receptors that might be involved. CR3 seemed a likely candidate because it has been shown to be capable of transducing LPS signals (25, 32), and flow cytometry results demonstrated that whereas THP-1rsv cells were deficient in CD14, they did express significant amounts of CR3 (Fig. 1). Indeed, blocking antibody experiments using THP-1rsv cells (CD14⁻/CR3⁺) clearly showed that CR3 was required for the late phase IRAK degradation (Fig. 6A). Similar blocking exper-
Exposure to LPS, PI 3-kinase becomes activated downstream of TLR4 and CR3. Thus, these findings suggest a model in which upon exposure to LPS, PI 3-kinase activity was assayed as described under "Experimental Procedures." Spots corresponding to phosphatidylinositol phosphate were cut and analyzed by scintillation counting. Activities are expressed as-fold increase with reference to control (untreated) cells. To ensure that equivalent amounts of PI 3-kinase were immunoprecipitated from experimental groups, 10% from each treatment sample was collected during the last wash in a separate tube and analyzed by SDS-PAGE and immunoblotting with Abs to the p85 PI 3-kinase subunit. The results shown are from one of two experiments that yielded similar results.

rations using both THP-1rv and THP-1wt showed that CR3 functioned independently of TLR4 in signaling delayed IRAK degradation. This result is consistent with the fact that in contrast to CD14, CR3 possesses a cytoplasmic domain through which signaling may proceed, independent of a transmembrane, signaling coreceptor.

The finding that dual receptors appeared to be involved in invoking independent pathways of IRAK degradation raised the important question of how these pathways were regulated. In examining this question, an important lead was provided by the findings that PKC-ζ associated with IRAK in LPS-treated THP-1 cells and the demonstration that PKC activity was required for LPS-induced IRAK degradation (40). These results, indicating a possible role for PKC-ζ in a pathway regulating IRAK degradation, led us to examine whether this may be regulated by PI 3-kinase because LPS has been shown to activate this lipid kinase leading to activation of PKC-ζ (39). As can be seen in Fig. 4, use of two distinct PI 3-kinase inhibitors, LY294002 and wortmannin, showed that PI 3-kinase was required for the early, but not the late phase of IRAK degradation. Thus, these findings suggest a model in which upon exposure to LPS, PI 3-kinase becomes activated downstream of CD14/TLR4 and signals through a pathway possibly leading to activation of PKC-ζ to bring about the rapid degradation of IRAK (Fig. 9). Identification of other elements that may be involved in this pathway is presently under investigation. One potential candidate is phosphoinositide-dependent kinase type 1 because it is known to be activated by phosphatidylinositol 3,4,5-trisphosphate and 3,4-bisphosphate through its pleckstrin homology domain (56), and it has been shown to phosphorylate and activate PKC-ζ directly (57, 58).

The finding that CR3-dependent, delayed phase IRAK degradation did not involve PI 3-kinase raised the question of how this pathway was regulated. It was recently reported that IRAK degradation in murine macrophages in response to either LPS or mucin-like glycoprotein from Trypanosoma cruzi may involve activation of serine/threonine phosphatases such as PP1 and PP2A (59). However, using okadaic acid pretreatment to inhibit serine/threonine phosphatase activity, we have thus far been unable to block either early or late-phase IRAK degradation in response to LPS. Whether the delayed pathway of IRAK degradation involves recruitment of nonreceptor tyrosine kinases downstream of CR3 or alternative signaling modules is currently under investigation.

Given the potentially severe consequences that follow from uncontrolled responses to LPS, it makes sense teleologically that mammalian cells have evolved parallel, independent pathways leading to IRAK degradation and LPS tolerance. Whether one pathway is more important than the other in maintaining homeostasis and if so which one are at this point conjectural. However, there are at least two reasons to consider that the CD14 pathway may predominate, at least under some conditions. First, CD14 is clearly the dominant cell membrane-bind-

Fig. 8. CD14, but not CR3, is required for activation of the PI-3 kinase in response to LPS. Serum-starved (6 h) THP-1wt cells were incubated with either anti-CD11b mAb M1/70 or anti-CD14 mAb 28C5 (final concentration 20 μg/ml) or left in RPMI 1640. Cells were then either left untreated or treated with 500 ng/ml LPS for 10 min. Cell samples were centrifuged at 400 × g and 4 °C followed by detergent lysis and immunoprecipitation with anti-PI 3-kinase antibody. PI 3-kinase activity was assayed as described under "Experimental Procedures." Spots corresponding to phosphatidylinositol phosphate were cut and analyzed by scintillation counting. Activities are expressed as-fold increase with reference to control (untreated) cells. To ensure that equivalent amounts of PI 3-kinase were immunoprecipitated from experimental groups, 10% from each treatment sample was collected during the last wash in a separate tube and analyzed by SDS-PAGE and immunoblotting with Abs to the p85 PI 3-kinase subunit. The results shown are from one of two experiments that yielded similar results.

Fig. 9. Dual, independent pathways of IRAK degradation are initiated separately through CD14/TLR4 and CR3. Both CD14/TLR4 and CR3 have the capacity to initiate the innate immune response to LPS through IRAK-dependent pathways leading to activation of NF-κB-dependent gene transcription. Negative feedback is provided by dual, independent pathways also triggered through CD14/TLR4 and CR3 leading to IRAK degradation and attenuation of the inflammatory response. LPS signaling through CD14 and TLR4 leads to rapid IRAK degradation, and this is dependent upon PI 3-kinase (PK-ζ). In parallel, a delayed wave of IRAK degradation initiated by CR3 occurs independently of both TLR4 and PI 3-kinase. The latter pathway is likely to be of particular importance in cells that are CR3⁺ and which express little or no CD14.
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(62). Taken together, these observations suggest that in the absence of significant amounts of CD14, CR3 has the capacity to activate an IRAK-dependent pathway leading to activation of NF-κB. Given the central roles of neutrophils in innate resistance and of dendritic cells in orchestrating both the innate and acquired immune responses, the capacity to attenuate the inflammatory response to LPS through a CR3-activated pathway of IRAK degradation is likely to be of considerable importance to mamalian homeostasis.

In summary, regulation of cell activation by endotoxin is critically important to controlling the host response during Gram-negative infection. Although the roles of IRAK in the response to LPS and in endotoxin tolerance have been firmly established, (16–19) how IRAK expression is regulated during exposure to bacterial LPS has not been fully elucidated. The results presented above identify CD14/TLR4 and CR3 as dual receptors that appear to trigger IRAK degradation through distinct and independent pathways (Fig. 9). These findings provide a basis for a model that allows tolerance to LPS to be achieved through two independent pathways, which may be located in tissues where limiting amounts of serum and LPS-binding protein may restrict optimal tolerance induction through CD14.

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