Interaction of matrix with integrin receptors is required for optimal LPS-induced MAP kinase activation

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Monick, Martha M., Linda Powers, Noah Butler, Timur Yarovinsky, and Gary W. Hunninghake. Interaction of matrix with integrin receptors is required for optimal LPS-induced MAP kinase activation. Am J Physiol Lung Cell Mol Physiol 283: L390–L402, 2002. First published March 1, 2002; 10.1152/ajplung.00437.2001.—Exposure of macrophages to endotoxin [lipopolysaccharide (LPS)] results in a cascade of events resulting in the release of multiple inflammatory and anti-inflammatory mediators. The Toll-like receptor (TLR) 4 complex is the major receptor that mediates LPS signaling. However, there is evidence that other surface molecules may play a complementary role in the TLR-induced events. Integrin receptors are one class of receptors that have been linked to LPS signaling. This study investigates the role of macrophage integrin receptors in the activation of mitogen-activated protein (MAP) kinases by LPS. In conditions where macrophages were not permitted to adhere to matrix or a tissue culture surface, we found a decrease in LPS signaling as documented by a marked reduction in tyrosine phosphorylation of whole cell proteins. This was accompanied by a significant decrease in extracellular signal-regulated kinase and c-Jun NH2-terminal kinase MAP kinase activation. Inhibition of integrin signaling, with EDTA or RGD peptides, decreased LPS-induced MAP kinase activity. The functional consequence of blocking integrin signaling was demonstrated by decreased LPS-induced tumor necrosis factor-α production. These observations demonstrate that, in addition to the TLR receptor complex, optimal LPS signaling requires complementary signals from integrin receptors. Toll-like receptor; tumor necrosis factor; integrins; lipopolysaccharide; mitogen-activated protein

LIPOLYSACCHARIDE (LPS) plays a pivotal role in the innate immune response to gram-negative bacteria (31, 42). LPS signaling is initiated by an interaction between LPS and LPS-binding protein, allowing binding to CD14 and association with another cell membrane receptor, which contains an intracellular signaling domain. This other receptor has recently been determined to be Toll-like receptor (TLR) 4, and significant work has been done to define this complex (for review see Refs. 1, 17, 22). In addition to the TLR complex, a number of other membrane receptors have been proposed to play a role in LPS signaling. These include β2-integrins, a purinergic receptor (P2X7), moesin, triggering receptor expressed on myeloid cells-1, a recently described complex that includes heat shock proteins 90 and 70, complement receptor 4, and bone morphogenic protein (20, 23, 29, 36). Several previous studies have suggested a role for the integrin receptors in LPS signaling (12, 23, 29, 36). Recently, Perera et al. (29) showed a decreased LPS response [some cytokines, e.g., cyclooxygenase-2 and interleukin (IL)-12 p35, and a slight decrease in p38 activation] in peritoneal macrophages from CD11b (a major integrin chain in macrophages)-knockout mice (29). This study evaluates the link between adherence and LPS-induced mitogen-activated protein (MAP) kinase activation and tumor necrosis factor (TNF)-α production in a murine macrophage line.

Integrins are a family of cell surface glycoproteins (for review see Refs. 2, 3, 18). Each integrin is a heterodimer that contains an α- and a β-subunit. Each subunit has a large extracellular domain, a single membrane-spanning region, and a short cytoplasmic domain that contains binding sites for other proteins but does not itself have signaling capabilities. There are 16 known α-subunits and ≥8 β-subunits, which can make up a multitude of integrins. In macrophages, the main adhesion molecules are integrins of the β1- or β2-class (39, 45). The β2-integrin (α2β1, αMβ2, αXβ2, and αMβ2) family has been especially linked to macrophage and leukocyte cell adhesion and spreading (14, 15, 36). In particular, αMβ2-integrin has been linked to LPS signaling by evaluation of cells from an αM-knockout mouse (29).

Integrins link the extracellular matrix (ECM) to the actin cytoskeleton and transmit biochemical signals across the plasma membrane (7). As well as transmitting signals from the ECM, integrins can also be activated from signals generated intracellularly. For example, several studies have documented the intracellular activation of integrins after G protein-coupled receptor activation (25, 37, 38). This phenomenon has been described as “inside-out” signaling. This contrasts with the signal generated when an integrin binds to...
the ECM and signals are generated: “outside-in” signaling (18). It is our hypothesis that LPS/TLR signaling activates integrins, increasing adhesion and generating an outside-in signal that amplifies LPS activation of the extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) MAP kinases. This results in increased production of TNF-α, suggesting a role for integrin signaling in macrophage inflammatory responses (8, 26).

Integrin signaling has been linked to LPS-induced macrophage activation (12, 27, 29). In other systems, it has been shown that integrins and growth factor-dependent signals converge on the MAP kinases (11). The MAP kinase family includes the ERK MAP kinase, JNK, and p38. ERK is regulated by a variety of agents, including integrins, growth factors, cytokines, and LPS. MAP kinase activation follows a cascade of kinase activation [MAP kinase (MEK) kinase (MEKK)-MEK-ERK] and can be demonstrated by phosphorylation of the threonine-tyrosine consensus motif (46). It has not been demonstrated that maximal LPS MAP kinase signaling depends on recruitment of integrin receptors and that integrin-dependent signals (secondary to the TLR signal) amplify MAP kinase activity and ultimately affect maximal TNF-α release.

In this study we have evaluated the contribution of adherence-dependent MAP kinase activation compared with adherence-independent MAP kinase activation in the setting of LPS-exposed macrophages. We found a consistent but smaller LPS-induced ERK and JNK activation when cells were kept in suspension. This is linked to the production of inflammatory mediators, because inhibition of ERK or JNK by chemical inhibitors decreased LPS-induced TNF production. In addition, two blockers of integrin signaling, EDTA and soluble RGD peptides (36, 49), blocked optimal ERK and JNK activation. This finding is also linked to TNF production, because RGD peptides and EDTA decreased LPS-induced TNF production. The link with MAP kinase activation is confirmed by the decrease in LPS-induced TNF production with inhibition of ERK or JNK. From these observations, we conclude that LPS signaling to MAP kinases is decreased without the added contribution of integrin signaling. In addition, optimal cytokine production requires integrin signaling and the increased MAP kinase activation that accompanies adherence. We found also that adherence alone did not activate ERK, suggesting a possible inside-out signal from the TLR-4 complex to integrins. This inside-out signal would increase integrin activation and macrophage adherence. The added adherence would, in turn, provide an outside-in signal that could augment LPS-induced ERK or JNK activation. These observations lead us to propose that, in macrophages, integrins provide a complementary receptor to the already described TLR-4 complex and that both contributions are necessary for optimal signaling.

MATERIALS AND METHODS

Materials. Chemicals were obtained from Sigma Chemical (St. Louis, MO), LPS from List Biological Laboratories (Campbell, CA), the MEK inhibitor U-0126 and the JNK inhibitor SP-600125 from Calbiochem (San Diego, CA), protease inhibitors from Boehringer Mannheim (St. Louis, MO), nitrocellulose and ECL Plus from Amersham (Arlington Heights, IL), and SuperSignal West Femto from Pierce (Rockford, IL). Antibodies were obtained from various sources: antibodies to ERK, p38, JNK, focal adhesion kinase (FAK), and phosphotyrosine from Santa Cruz Biotechnology (Santa Cruz, CA), phosphorylation-specific antibodies to p38 from Sigma Chemical, and all other phosphorylation-specific antibodies, including ERK, JNK, c-Jun, and phosphotyrosine, from Cell Signaling (Beverly, MA). Developing antibodies (horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig) were obtained from Santa Cruz Biotechnology. Special adherence-free plates (Ultra Low Attachment) were obtained from Corning (Corning, NY) and Teflon tubes from VWR (Batavia, IL). RGDS (RGD) peptide (Arg-Gly-Asp-Ser) was obtained from Bachem (Torrance, CA).

Cell culture. RAW 264.7 (RAW) cells (TIB-71, American Type Culture Collection) were maintained in DMEM with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL amphotericin B). Cells were subcultured every 2–3 days. Experiments were run in six-well Costar tissue culture plates, low-adherence plates, or Teflon. For some experiments, cells were cultured in plates coated with fibronectin or collagen IV (BD Biocoat, Becton Dickinson Labware).

Isolation of whole cell extracts. RAW cells were cultured in various conditions. Whole cell protein was obtained by lysing the cells on ice for 20 min in 500 μl of lysis buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.5 M phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate; all from Boehringer Mannheim). The lysates were then sonicated for 20 s, kept at 4°C for 30 min, and spun at 15,000g for 10 min, and the supernatant was saved. Protein was determined using a protein measurement kit (protein assay kit 500-0006, Bio-Rad, Hercules, CA). Cell lysates were stored at −70°C until use.

Immunoprecipitation of proteins. RAW cells were cultured in various conditions, and whole cell proteins were obtained. Aliquots (300 μg) from each sample were placed in a new tube for immunoprecipitation. The volume was brought up to 500 μl with lysis buffer with added sodium orthovanadate (2 mM). Rabbit anti-FAK antibody (20 μl/sample) was added, and the samples were rotated at 4°C overnight. On the next day, protein A-agarose (20 μl/sample; Santa Cruz Biotechnology) was added to each sample, and the tubes were rotated at 4°C for 1 h. The beads were subsequently washed four times with lysis buffer with added sodium orthovanadate (2 mM). The immunoprecipitated complexes were released with 2× sample buffer for Western analysis.

Western analysis. Western analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed on whole cell proteins from RAW cell experiments. Protein (30–80 μg) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, and 1.25 M Tris, pH 6.8; all from Sigma Chemical) heated to 95°C for 5 min, loaded onto a 10% SDS-polyacrylamide gel, and run at 100 V for 90 min. Cell proteins were transferred to nitrocellulose (ECL, Amersham) by semidy transfer (Bio-Rad) at 25 V for 45 min. Equal loading of the protein groups on the blots was evaluated by using Ponceau S, a staining solution designed for staining proteins on nitrocellulose membranes (Sigma Chemical) or, in the case of phosphorylation-specific blots, by stripping and reprobing with antibodies to the total protein. The nitrocel-
lulose was blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 for 1 h, washed, and then incubated with the primary antibody overnight. The blots were washed four times with Tris-buffered saline with Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody. Immunoreactive bands were developed using a chemiluminescent substrate (ECL Plus or SuperSignal West Femto). An autoradiograph was obtained, with exposure times of 10 s–2 min.

Adherence assay. RAW cells were cultured in adherent or nonadherent conditions in special 24-well plates (10⁶ cells/well; triplicate wells per group); some cells were kept aside for use in a standard curve. After the culture period, the wells were washed three times with prewarmed PBS with calcium and magnesium (0.5 ml/well; wide-bore pipette tips were used to add PBS), and the plate was blotted on thick blotting paper. PBS (0.5 ml) was added to each well along with a standard curve of cells in 0.5 ml PBS/well (10⁶–10⁵ cells/well). Calcein-AM (0.5 ml, 2 μM final concentration; Molecular Probes) was added to each well. The plate was incubated at 37°C for 30 min, and fluorescence was measured using a 485 ± 8 nm excitation filter and a 535 ± 10 nm emission filter on a Victor® (EG & G Wallac, Gaithersburg, MD) microplate reader. Adherence was defined as the percentage of cells remaining in the plate after the PBS washes. Cell numbers were determined by comparing sample values with the standard curve generated by the known amount of cells.

Cytokine release. RAW cells were cultured in standard medium for 6 h in adherent and nonadherent conditions with and without LPS. After the culture period, the supernatants were harvested and stored at −70°C until assayed. The amount of TNF-α in the supernatant was measured by ELISA (R & D Systems, Minneapolis, MN).

Isolation of RNA. Total RNA was isolated using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. RNA was quantitated using the RiboGreen kit (Molecular Probes). RNA samples were stored at −70°C.

RT-PCR detection of TNF-α mRNA. Total RNA (1 μg) was reversed transcribed to cDNA using the RETROscript RT-PCR kit (Ambion, Austin, TX). The resulting cDNA was subjected to PCR as follows. In a 0.2-ml PCR tube (Bio-Rad), 2 μl of cDNA were added to 48 μl of PCR mixture containing dNTP (Life Technologies, Grand Island, NY) at 2 mM each, 1.5 mM MgCl₂ (Life Technologies), 1:15,000 SYBR Green I DNA dye (Molecular Probes), sense and antisense primers (Research Genetics, Huntsville, AL) at 0.2 μM each, and 2.5 units of platinum Taq DNA (Life Technologies). Amplification was then performed in an iCycler iQ Fluorescence thermocycler (Bio-Rad) as follows: 3 min at 95°C, followed by 45 cycles of 20 s at 95°C, 20 s at 59°C, 20 s at 72°C, and 10 s at 81°C. Fluorescence data were captured during the dwell at 81°C. Data were collected and recorded by iCycler iQ software (Bio-Rad) and expressed as a function of threshold cycle (C₀), the cycle at which the fluorescence intensity in a given reaction tube rises above background. Primers for murine TNF-α and hypoxanthine phosphoribosyltransferase (HPRT) genes are as follows (5’ to 3’): AACTTCGGGGTGATCG-CAATTCGGGGTGATCG (sense) and CAAATCGGCTGACGGTGTGGG (antisense)...

Fig. 1. Low adherence prevents cell attachment to plates and decreases lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α activation of macrophages. RAW cells were cultured in normal tissue culture plates (Adherence) and special low-adherence plates (Low Adherence) for 30 min. A: photomicrograph demonstrates increased binding and clumping of cells in adherence plate. Differences in adherence are documented by an adherence assay (see MATERIALS AND METHODS). B and C: low adherence decreases LPS-induced TNF-α mRNA and protein production. RAW cells were cultured in adherence and low-adherence conditions for 30 min and then treated with LPS (1 μg/ml) for 3 h (mRNA) or 6 h (protein). RNA was isolated according to the protocol described in MATERIALS AND METHODS, and relative amounts of RNA were determined by real-time PCR. Data are normalized to hypoxanthine phosphoribosyltransferase (HPRT) and presented as fold increase compared with adherence control cells. Data represent a composite of 3 experiments. TNF-α release was determined by harvesting culture supernatants and evaluating TNF-α amounts by ELISA. mRNA and ELISA data represent 3 experiments.
HPRT were determined. The TNF-α/H9251 function was determined by Student densitometry data, ELISA results, and real-time PCR data. Primers were found to be identical.

The amplification efficiencies for TNF-α and HPRT genes. With the use of this set of template mixtures, control TNF-α serial 10-fold dilutions of template containing TNF-α/H9251 were captured at 3°C below the lowest melting temperature among all amplimers assayed to ensure that primer-dimers were not contributing to the fluorescence signal generated with SYBR Green I DNA dye. Specificity of the amplification was confirmed using melting curve analysis.

**Quantification of TNF-α mRNA.** Relative quantitative gene expression was calculated as follows. For each sample assayed, the C_t values for reactions amplifying TNF-α and HPRT were determined. The TNF-α C_t for each sample was corrected by subtracting the C_t for HPRT (ΔC_t). Untreated controls were chosen as the reference samples, and the ΔC_t values for all LPS-treated experimental samples were subtracted from the ΔC_t values for the control samples (ΔΔC_t). Finally, LPS-treated TNF-α mRNA abundance relative to control TNF-α mRNA abundance was calculated as follows: 2^(ΔΔC_t). The validity of this approach was confirmed by using serial 10-fold dilutions of template containing TNF-α and HPRT genes. With the use of this set of template mixtures, the amplification efficiencies for TNF-α and HPRT amplimers were found to be identical.

**Statistical analysis.** Statistical analysis was performed on densitometry data, ELISA results, and real-time PCR data. Significance was determined by Student’s t-test.

**RESULTS**

**LPS requires an adherence-induced signal for maximal TNF-α production.** We initially validated our low-adherence model by a specific adherence assay. Using RAW cells, we cultured cells for 30 min in a standard tissue culture plate (adherence) or in a special low-adherence plate with and without LPS (1 μg/ml). Photomicrographs were obtained, and an adherence assay was performed (see MATERIALS AND METHODS). Figure 1A shows RAW cells in the adherence and low-adherence conditions. Under low-adherence conditions, the cells remain rounded and distributed in a single-cell manner. In contrast, the adherence cells were clumped together and beginning to stick to the plate. The degree of adherence in the two plates after 30 min is quantified and shown in Fig. 1A. We also evaluated total tyrosine phosphorylation of whole cell proteins and found that low adherence decreased the LPS-induced tyrosine phosphorylation in a number of bands (data not shown). This suggests a role for integrin signaling in LPS macrophage responses. Exposure of macrophages to LPS starts a chain of events culminating in the release of multiple inflammatory and anti-inflammatory mediators. Central to macrophage immune function is the release of TNF-α (13, 33). Because of the importance of TNF-α, we wanted to evaluate whether the decreased signaling exemplified by the lowered tyrosine phosphorylation in low-adherence conditions had an effect on TNF-α production. RAW cells were cultured in low-adherence and adherence conditions. LPS was added for 3 h (RNA) or 6 h (protein). Relative TNF-α RNA levels were determined using real-time RT-PCR (see MATERIALS AND METHODS), and protein release was determined by ELISA. Figure 1B demonstrates that preventing adherence during LPS exposure results in decreased production of the proinflammatory cytokine TNF-α.

**MAP Kinase Activity**

![MAP Kinase Activity Image]

**Densitometry**

![Densitometry Image]

**Fig. 2.** Low adherence decreases LPS-induced mitogen-activated protein (MAP) kinase activation. RAW cells were cultured in adherence and low-adherence conditions for 30 min and then treated with LPS (1 μg/ml) for 5 (5'), 15 (15'), and 60 (60') min. Whole cell protein was obtained, and Western analysis was performed using antibodies specific for phosphorylated extracellular signal-regulated kinase (phospho-ERK), c-Jun NH2-terminal kinase (phospho-JNK), and p38 (phospho-p38). Blots were then stripped and reprobed for total protein amounts. Primary antibody concentrations of 1:500 and secondary antibody concentrations of 1:10,000 were used. Immunoreactive bands were visualized using chemiluminescence. Densitometry of phosphorylated MAP kinases is shown as fold increase [mean optical density (OD) units in control sample/mean OD units in experimental sample]. Results are representative of 3 experiments.
LPS-induced MAP kinase activation is decreased in low-adherence conditions. Activation of multiple MAP kinases has been linked to LPS-induced TNF-α production (8, 26). We next evaluated whether lack of adherence correlated with a decrease in MAP kinase activation by LPS. RAW cells were cultured in adherence vs. low-adherence conditions with and without LPS. Whole cell lysates were obtained at various times after LPS and analyzed for MAP kinase activation using antibodies specific for the phosphorylated form of ERK, JNK, and p38. Phosphorylation at the tyrosine-threonine motif in these MAP kinases is essential for activation. Figure 2 shows a significant decrease in the activation of all three MAP kinases when LPS exposure occurred in the low-adherence conditions. The smallest decrease was observed in the p38 blot. In addition, over multiple experiments the observation that low adherence decreased LPS-induced p38 activation was not consistent (data not shown). This suggests a tenuous link between integrin signaling and LPS-induced p38 activation. Because of the solid link between adherence and optimal ERK and JNK activation by LPS, we concentrated on these kinases in the remaining experiments. The data demonstrate that, over an extended time course, low adherence decreases the degree of MAP kinase activation in RAW cells after LPS exposure.

**ERK activation is linked to LPS-induced TNF production.** To determine that ERK activation was relevant to the decreases in TNF production in low-adherence cells, we evaluated TNF production after inhibition of ERK activity with the inhibitor U-0126. U-0126 prevents ERK activation downstream of MEK (43) and is a more complete blocker of ERK activation than the commonly used PD-98059. We found that U-0126 almost completely blocked LPS-induced TNF in adherence and low-adherence conditions (Fig. 3). Low adherence does not completely block ERK activation, which explains the difference in degree of TNF inhibition between U-0126 and low adherence alone. As controls, we also demonstrated that U-0126 does block ERK activation and does not block JNK activa-
tion or the tyrosine phosphorylation of FAK, an integrin-linked kinase.

**JNK activation is linked to LPS-induced TNF production.** To determine that JNK activation was relevant to the decreases in TNF production seen in low-adherence cells, we evaluated TNF production after inhibition of JNK activity with the inhibitor SP-600125, which prevents JNK activity (6). We found that SP-600125 decreases LPS-induced TNF (Fig. 4). As controls, we also demonstrated that SP-600125 does not block ERK activation and does block JNK activation using phosphorylation of the JNK substrate c-Jun as a readout. SP-600125 inhibits the catalytic activity of JNK and not the activating phosphorylations. For this reason, the phosphorylation-specific JNK antibody cannot be used to measure JNK activity after SP-600125. c-Jun is phosphorylated on Ser 73 by JNK and can be used to monitor JNK activity.

**Increasing the length of adherence before LPS exposure does not activate ERK.** RAW cells were cultured in adherence vs. low-adherence conditions for various time periods before the addition of LPS. After exposure to LPS for 15 min, whole cell lysates were obtained, and ERK phosphorylation was evaluated. Figure 5 demonstrates that adherence alone does not activate ERK in RAW cells. LPS is needed for ERK activation, and LPS plus adherence is needed for optimal ERK activation.

**LPS activation of ERK does not change on different ECM.** We next asked the question: Is LPS-induced ERK activation dependent on a particular substrate? RAW cells were placed in culture for 30 min in adherence plates, low-adherence plates, fibronectin-coated plates, or collagen IV-coated plates. The cells were treated with LPS for 15 min, whole cell protein was obtained, and ERK phosphorylation was evaluated. Figure 6 shows an increase in ERK phosphorylation (activity) in the adherence plates similar to that in plates coated with fibronectin or collagen IV. This study suggests that binding matrices other than the plastic tissue culture dish (fibronectin and collagen IV) can also play a positive role in LPS-induced ERK activation.

**LPS-induced tyrosine phosphorylation of FAK depends on adherence.** In other systems, integrin involvement in MAP kinase activation is via FAK (4, 35). To
determine the effect of adherence on FAK tyrosine phosphorylation (a marker of activity) in RAW cells, we treated cells with LPS for various periods of time and immunoprecipitated FAK. The immunoprecipitated FAK was then evaluated for tyrosine phosphorylation with an antibody that recognizes phosphotyrosine (4G10). Figure 7 demonstrates that LPS increases tyrosine phosphorylation of FAK and that the low-adherence conditions prevented even the baseline FAK phosphorylation. The blot was stripped and reprobed for total FAK. There is a decrease in the amount of total FAK immunoprecipitated in the adherence conditions because of the difficulty of isolating proteins that are part of the detergent-resistant membrane (activated FAK). However, this difference only makes the lack of tyrosine phosphorylation in the low-adherence conditions more impressive. The data suggest a link between LPS signaling and integrin activation and also suggest that FAK activity might contribute to LPS-induced MAP kinase activation.

To confirm the link between FAK activation and LPS-induced ERK activity, we treated the cells with cytochalasin D for 2 h before adding LPS. Cytochalasin D disrupts actin polymerization, which is necessary for FAK phosphorylation (16, 34). It has been used to demonstrate a link between FAK activation and downstream signaling in other studies (16). Figure 7B demonstrates that disrupting actin polymerization via cytochalasin D decreases ERK activation by ~50%, which suggests that FAK signaling is one, but not the only, pathway to LPS-induced ERK activation.

Adherence-dependent LPS activation of ERK and JNK is blocked by added EDTA. Integrin signaling requires Ca2+ and in experimental models of integrin signaling the addition of EDTA to the extracellular medium has been used as an integrin inhibitor (49). To confirm that the decrease in signaling in the low-adherence condition is mediated by integrin, we treated RAW cells with EDTA for 15 min before adding LPS. Figure 8A demonstrates that the increased ERK and JNK phosphorylation in adherence plates was blocked by the preaddition of EDTA. There was also a small decrease in ERK activation in the low-adherence samples, perhaps because of a role of extracellular Ca2+ in TLR-4 signaling or a small amount of integrin signaling generated by cell-to-cell contact in the low-
adherence plates. To link the integrin-dependent signals to LPS-induced TNF production, we evaluated LPS-induced TNF production in cells pretreated with EDTA. Figure 8B shows that EDTA decreases LPS-induced TNF production in mouse macrophages.

Adherence-dependent LPS activation of ERK and JNK is blocked by added RGD peptides. Cell attachment via integrins is mediated through a recognition site on the extracellular portion of the integrin heterodimer that recognizes a tripeptide, RGD, in target proteins of the ECM (32). Soluble peptides containing the RGD motif can be used to inhibit cell attachment (9). To further link optimal LPS signaling to integrin engagement, we treated RAW cells with synthetic RGD peptides 15 min before the cells were plated on adherence plates. The cells were cultured for 30 min, and then LPS was added for 15 min. Figure 9A shows that the RGD peptides keep the cells separate, in contrast to the LPS-treated cells, which demonstrate increased clumping and sticking. Figure 9B quantifies this using an adherence assay. This assay demonstrates that the RGD-treated cells do not demonstrate the increase in adherence that is found in the LPS-treated cells. Figure 9C shows that pretreatment with RGD peptides decreases ERK and JNK phosphorylation. This correlates with the cells' ability to produce TNF-α, because addition of the RGD peptides significantly decreases the amount of TNF-α produced by LPS-treated cells (Fig. 9D). The data shown here, when considered in conjunction with the previous experiment utilizing EDTA, demonstrate a strong link between increased ERK and JNK, TNF-α production, LPS-induced activation, and integrin signaling.

DISCUSSION

The premise of this investigation was that LPS signaling to MAP kinases would be significantly enhanced by cooperation between the TLR-4 complex and integrin receptors. To investigate this, we utilized RAW
cells, a murine macrophage line, to study the effect of integrin-mediated signal on LPS activation of MAP kinases. We found that maintaining the cells in suspension (low adherence) significantly decreased the degree of LPS activation of macrophages. We initially correlated lack of cell adherence with a decrease in the amount of TNF-α produced by LPS-treated cells. Because of the decrease, we then evaluated the effect of low adherence on MAP kinase activation and found that all three MAP kinases exhibited decreased activation in low adherence. This was especially the case for ERK and JNK, and the remaining experiments evaluated these MAP kinases. The increase in MAP kinase activity was mirrored by an increase in FAK tyrosine phosphorylation that was completely absent in the low-adherence conditions. We linked the adherence-dependent FAK phosphorylation to LPS-induced ERK activation by showing that cytochalasin D disrupted LPS-induced ERK activation. Two blockers of integrin signaling, EDTA and soluble RGD peptides, blocked the increased MAP kinase activation demonstrated in adherence plates. RGD peptide and EDTA decreases in adherence translated into less LPS-induced TNF-α. In addition, we found that ERK and JNK activity was linked to optimal TNF production after LPS exposure. As an aggregate, the data suggest that, in order for LPS to induce a maximal response in macrophages, there must be cooperation between the TLR complex and integrin signaling (Fig. 10).

Integrin activation of ERK has been documented in other systems (14). ERK is activated when serum-starved fibroblasts are placed on fibronectin (19). Epidermal growth factor activation of ERK requires adherence (30). How integrins are linked to activation of MEK and then ERK is not clear. Integrins are known to activate a major tyrosine kinase pathway initiated...
Fig. 9. RGD peptides block RAW cell adherence, resulting in decreased MAP kinase activation and TNF-α production. RAW cells were cultured in adherence conditions for 30 min and then treated with LPS (1 μg/ml) with and without RGD peptides (1 mM). RGD peptides were added immediately before cells were plated. A: photomicrograph showing rounded unclumped cells in RGD-treated groups and progressively more clumpy and adherent cells in groups treated with LPS alone. Arrows point to progressively larger clumps. B: percentage of adherent cells in groups in A. C: RAW cells were treated with LPS (1 μg/ml) for 15 min, whole cell lysates were obtained, and Western analysis was performed using antibodies specific for phosphorylated ERK. Blots were then stripped and reprobed for total protein amounts. Primary antibody concentrations of 1:500 and secondary antibody concentrations of 1:10,000 were used. Immunoreactive bands were visualized using chemiluminescence. D: RAW cells were cultured in adherence conditions for 30 min and then treated with LPS (0–1,000 ng/ml) with and without RGD peptides (1 mM) for 6 h. Supernatants were harvested, and TNF-α production was analyzed by ELISA. Data are representative of 3 experiments.
with activation of FAK (5). FAK undergoes autophosphorylation at Tyr925, which results in the recruitment of Src. This results in the phosphorylation of paxillin and p130Cas, which serve as adaptors for the recruitment of other signaling molecules (35). One event that occurs during this cascade is phosphorylation of FAK on Tyr925, creating a binding site for the adaptor protein Grb2. Grb2 recruits Sos and activates the classic Ras-Raf-MEK-ERK cascade (4, 35). However, alternative studies have demonstrated FAK-independent integrin activation of ERK (24, 44) as well as Ras-independent integrin activation of ERK (10). Our data do not address the specific pathway activated by integrin signaling, but they do suggest a role for integrins and FAK in LPS-mediated signaling. Further studies will determine the exact nature of the downstream effectors involved in LPS/integrin/ERK signaling.

Cross talk between integrins and other receptors has been documented in a number of cases. In neutrophils, the CD11b/CD18 integrin receptor cooperates with the FcγRIII to generate a respiratory burst (48). Using carcinoma cells, Yebra et al. (47) found that α2β3-integrin-directed cell migration required activation of the urokinase-type plasminogen activator receptor. In this study, cell migration on vitronectin was blocked by blocking antibodies to the urokinase-type plasminogen activator or the integrin receptor α2β3. This suggests a required cross talk between the two receptors. In a T cell model of migration through endothelium, Hwang et al. (21) found that signaling via the glycosylation-dependent cell adhesion molecule-1 receptor (an L-selectin receptor) increased the avidity of β2-integrins, allowing for T cell recruitment into peripheral lymphoid tissue. This model is similar to our hypothesis for TLR-4 and integrin signaling: LPS/TLR-4 provides a signal that increases integrin affinity, resulting in adherence to ECM, further activating the integrins, which then provide an enhanced signal to MAP kinases.

Our hypothesis is that the sequence of events leading to optimal ERK and JNK activation in macrophages is as follows: LPS activation of the TLR-4 complex → inside-out activation of an integrin receptor → increased adherence → integrin signaling outside-in to MAP kinases. A recent study by Schmidt et al. (36) supports the inside-out part of this story. They found that, in J774.A1 macrophages, LPS activated the β2-chain of the integrin family via a sequential activation of TLR-4-myeloid differentiation factor-88-IL-1 receptor-associated kinase-p38-Rap1 GTPase. This LPS-induced pathway results in cell spreading. In support of the outside-in signaling, Perera et al. (29) showed that macrophages from mice deficient in CD11b/CD18 (or α2β3) produced decreased amounts of some cytokines (cyclooxygenase-2 and IL-12 p35) in response to LPS or taxol. However, they found no difference in the expression of TNF-α. The fact that the knockout in question was to the CD11b chain leaves the possibility of other β2-heterodimers contributing to the TNF-α-inducing signal. One important difference between our study and that of Perera et al. is that their system blocks only one of the integrin α-chains, while our system blocks all integrin signaling. Their study does support the role of integrins in LPS signaling, inasmuch as they found decreased NF-xB translocation, decreased p38, and decreased cytokines in the CD11b knockouts. The differences they described were substantially greater in CD14- and TLR-4-knockout macrophages, suggesting that the TLR-4 complex is the primary receptor complex.

The regulation of multiple cellular events after macrophage LPS exposure is complex. Because of the extreme bioactivity of many of the molecules produced by the macrophage (i.e., TNF-α), multiple checkpoints are in place. One possible checkpoint is the interaction of multiple receptors. Our data (lack of MAP kinase activation with adherence but augmented LPS-induced MAP kinase activation with adherence) suggest that the TLR-4 complex interacts with integrin receptors. This suggests that optimal LPS responses are regulated at many levels, including that of a multiplicity of receptors.

This study demonstrates that optimal LPS signaling, to important signaling molecules (MAP kinases) and ultimately to the generation of inflammatory mediators (TNF), requires the cooperation of integrin receptors. Although a role for integrin receptors in LPS responses has been suggested, this study establishes a unique link between integrins and MAP kinase activation in the context of LPS stimulation. We believe that this is an important addition to the study of inflammation.
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