Polymeronuclear leukocytes (neutrophils) respond to lipopolysaccharide (LPS) through the up-regulation of several pro-inflammatory mediators. We have recently shown that LPS-stimulated neutrophils express monocyte chemotactic protein 1 (MCP-1), an AP-1-dependent gene, suggesting that LPS activates the c-Jun N-terminal kinase (JNK) pathway in neutrophils. Previously, we have shown the activation of p38 MAPK, but not JNK, in suspended neutrophils stimulated with LPS but have recently shown activation of JNK by TNF-α in an adherent neutrophil system. We show here that exposure to LPS activates JNK in non-suspended neutrophils and that LPS-induced MCP-1 expression, but not tumor necrosis factor-α (TNF-α) or interleukin-8 (IL-8), is dependent on JNK activation. In addition, LPS stimulation of non-suspended neutrophils activates Syk and phosphatidylinositol 3-kinase (PI3K). Inhibition of Syk with piceatannol or PI3K with wortmannin inhibited LPS-induced JNK activation and decreased MCP-1 expression after exposure to LPS, suggesting that both Syk and PI3K reside in a signaling pathway leading to LPS-induced JNK activation in neutrophils. This Syk- and PI3K-dependent pathway leading to JNK activation after LPS exposure in non-suspended neutrophils is specific for JNK, because inhibition of neither Syk nor PI3K decreased p38 activation after LPS stimulation. Furthermore, we show that PI3K inhibition decreased LPS-induced Syk activation suggesting that PI3K resides upstream of Syk in this pathway. Finally, we show that Syk associates with Toll-like receptor 4 (TLR4) upon LPS stimulation further implicating Syk in the LPS-induced signaling pathway in neutrophils. Overall, our data suggest that LPS induces JNK activation only in non-suspended neutrophils, which proceeds through Syk- and PI3K-dependent pathways, and that JNK activation is important for LPS-induced MCP-1 expression but not for TNF-α or IL-8 expression.

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References

1. This paper is available on line at http://www.jbc.org

2. The abbreviations used are: LPS, lipopolysaccharide; KRPD, Kreb’s Ringer phosphate buffer with dextrose; HIPPP, heat-inactivated platelet poor plasma; DFP, diisopropyl fluorophosphate; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; TLR, Toll-like receptor; MCP-1, monocyte chemoattractant protein; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; IL-1, interleukin-1; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; MKK, MAP kinase kinase.
presence of pathways independent of MyD88 and Mal/Tirap, which lead to the activation of the MAPKs after LPS stimulation. PI3K and the small G-proteins Cdc42 and Rac1, which are involved in the activation of MAPKs (19–31), particularly JNK (26–30, 32–35), have been demonstrated to associate with TLR2 in response to heat killed *Staphylococcus aureus* (36). Whether PI3K and/or the small G-proteins might be involved in TLR4-dependent activation of the MAPKs after LPS stimulation, and what the functional consequences of such activation, is unknown.

The JNK subfamily of MAPK has been shown to be involved in a variety of cellular functions. Activation of JNK has been linked to induction of apoptosis, as seen after TNF-α (37) and IL-1β stimulation (38), and has been suggested to be important in the transcripational regulation of several inflammatory mediators, including IL-2 (39), Cox-2 (40), metalloproteinase 1 (39), vascular endothelial growth factor (41), and monocyte chemoattractant protein 1 (MCP-1) (39). JNK is activated upon exposure to osmotic stress (42), irradiation (43), growth factors (44), heat shock (42), IL-1β (39), and TNF-α (37, 42, 45).

In addition, JNK activation has been shown to occur in macrophages upon LPS stimulation (46–48), however, the activation of JNK in LPS-stimulated neutrophils has not been previously described. We (49, 50) and others (51) have reported that LPS stimulation of suspended neutrophils results in the activation of p38 MAPK, but not ERK or JNK. The absence of JNK activation in our previous studies may relate to the use of suspended neutrophils, which might not replicate events that occur in a complex microenvironment, because we have recently shown the activation of JNK in adherent neutrophils upon TNF-α stimulation (37).

Several pathways leading to JNK activation have been described, which, although stimulus-dependent, display common features. Pathways have been described that depend on the small G-proteins Cdc42 and Rac1 (30, 31), PI3K (34, 43, 44), and recently we have identified Syk as an upstream activator of JNK in adherent neutrophils after TNF-α stimulation (37). Activation of JNK after LPS stimulation in macrophages requires PI3K (46), although upstream components in the JNK pathway in neutrophils are unknown. We queried whether activation of Syk and PI3K may also occur in LPS-stimulated neutrophils, and if so whether Syk and PI3K participate in LPS-induced JNK activation.

We show here that LPS stimulation activates JNK in non-suspended neutrophils and that this activation occurs through Syk-dependent and both PI3K-dependent and -independent pathways. Additionally, we show that LPS stimulates PI3K and Syk activation, that PI3K is involved in the activation of Syk, and that Syk associates with TLR4 upon LPS stimulation. This pathway leading to the activation of JNK in non-suspended neutrophils appears independent of that which leads to p38 activation after LPS stimulation. Finally, we show that inhibition of JNK leads to a relatively specific decrease in LPS-induced MCP-1 expression in neutrophils. These studies provide new insight into mechanisms by which neutrophils respond to LPS.

**Experimental Procedures**

**Materials**—All reagents and plasticware used in these experiments were endotoxin-free. Leupeptin, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), EDTA, diisopropyl fluorophosphate (DFP), aprotinin, wortmannin, Brij 97, protein A-Sepharose, and RedTag polymerase were purchased from Sigma (St. Louis, MO). Lipopolysaccharide (*Escherichia coli* 0111:B4) was purchased from List Biological Laboratories (Campbell, CA). γ-[32P]ATP was purchased from Amersham Biosciences (Arlington Heights, IL). TRIzol and Moloney murine leukemia virus-reverse transcriptase were purchased from Invitrogen (Grand Island, NY), and piceatannol and the JNK inhibitor II (SP600125) were purchased from Calbiochem Biochemicals (La Jolla, CA). Antibodies to JNK-1 (H-80, HTA125, and C-18), JNK-2 (C92), Syk (C20), Syk (N19), and Hck were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the antibody to p85 of PI3K and the anti-phosphotyrosine (clone G410) agarose conjugate were purchased from Upstate Biotechnology Inc. (Lake Placid, NY), the phospho-Syk (Tyr-352) antibody was purchased from Cell Signaling Technology (Beverly, MA), the Lyn antibody was purchased from BD Transduction Laboratories (San Diego, CA), and the antibody against p38 was prepared as previously described (49). The substrates c-Jun-1, -9, and ATF-2, -11 were generated as previously described (49).

**Human Neutrophil Isolation**—Human neutrophils were isolated from healthy donors as described previously (49, 52). The isolation medium utilized is a combination of 99% cell viability with 10% contaminating monocytes (49). Neutrophils were isolated from peripheral blood from healthy volunteers, stimulated with LPS (200 ng/ml), and kept at 37 °C for 60 min under non-suspended conditions undisturbed by the cells were then stimlated with LPS (200 ng/ml) for 2 h at 4 °C by agitation.

**Immunoprecipitation**—Isolated human neutrophils (20 × 10^6/ml were used throughout) were resuspended and placed into microcentrifuge tubes in complete KRPD with 1% HIPPP and protease inhibitors and kept at 37 °C for 60 min under non-suspended conditions undisturbed by cell agitation. The cells were then stimulated with LPS (200 ng/ml) for 2 h at 4 °C by agitation.

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LPS Induces JNK Activation

Statistical Analysis—Data are expressed as means ± S.E. Multiple comparisons were performed by one-way ANOVA with Tukey (post hoc) test for determination of differences between groups. Statistical analysis was also performed by Student’s paired \( t \) test where indicated. A \( p \) value less than 0.05 was considered significant.

RESULTS

LPS Induces the Activation of c-Jun NH\(_2\)-terminal Kinase (JNK) in Neutrophils—Because we have recently observed increased expression of MCP-1 mRNA upon LPS stimulation in neutrophils (54), and the induction of AP-1, of which c-Jun is one component, is important for the expression of MCP-1 mRNA (55, 56), we hypothesized that LPS may induce the activation of JNK. Isolated human neutrophils were incubated under non-suspended conditions for 60 min, then exposed to LPS, followed by immunoprecipitation of JNK1 and analysis by radiobinding assay using recombinant c-Jun1–79 as an exogenous substrate. As can be seen in Fig. 1, LPS induced an increase in JNK activity, as indicated by increased phosphorylation of the c-Jun fragment c-Jun\(_{1–79}\) as early as 10 min after exposure to LPS, which peaked at 15 min, and was maintained for at least 30 min after LPS stimulation. The LPS-induced activation of JNK occurs more rapidly than compared with the activation of p38 seen previously, which peaks 30 min after stimulation by LPS (49).

The JNK Inhibitor SP600125 Inhibits LPS-induced JNK Activation—To assess the effectiveness of the JNK inhibitor SP600125 on LPS-induced JNK activation in neutrophils, human neutrophils were pretreated for 60 min with increasing concentrations of SP600125 prior to LPS stimulation, followed by an in vitro JNK kinase assay. As can be seen in Fig. 2, prior treatment with SP600125 diminished JNK activation in a dose-dependent manner with a complete inhibition of the LPS-induced increase in JNK activation at a concentration of 5 \( \mu \)M (Fig. 2). To assess the specificity of SP600125 on LPS-induced JNK activation, and in particular to confirm that SP600125 did not affect activation of p38, the other MAPK activated in LPS-stimulated neutrophils, we assessed the LPS-induced activation of p38 in neutrophils pretreated with SP600125 (2–10 \( \mu \)M) prior to LPS stimulation. Activation of p38 after LPS stimulation was not diminished by pretreatment with SP600125 at any concentration utilized (data not shown).

Effect of JNK Inhibition on LPS-induced Cytokine and Chemokine Expression—Neutrophils respond to LPS with increased expression of TNF-\( \alpha \), IL-8, IL-1\( \beta \), IL-6, and macrophage inflammatory protein 1\( \alpha \) (2, 3, 57). Recently we have shown that in addition to the up-regulation of these pro-inflammatory mediators, neutrophils have increased expression and release of MCP-1 after LPS stimulation (54). The transcriptional regulation of MCP-1 mRNA expression has been shown to be controlled primarily through the activation of AP-1, of which c-Jun is a prototypical component (55, 56). To examine the downstream effects of JNK inhibition on LPS-induced cytokine and chemokine expression, neutrophils were incubated for 60 min with the JNK inhibitor SP600125 (2–20 \( \mu \)M), stimulated with LPS for 4 h, followed by assessment of mRNA expression by RT-PCR and protein release by ELISA. As shown in Fig. 3A, pretreatment with 2 \( \mu \)M SP600125 prior to LPS stimulation resulted in a decrease in MCP-1 mRNA expression with a complete inhibition of LPS-induced MCP-1 expression at concentrations of 5 \( \mu \)M and greater of SP600125. In contrast
FIG. 2. The JNK inhibitor SP600125 inhibits LPS-induced JNK activation. Neutrophils were incubated with increasing concentrations of SP600125 (2–20 μM), or Me₂SO (0.1%) as diluent control, for 60 min at 37 °C followed by stimulation with LPS (100 ng/ml) for 4 h. RT-PCR was performed on extracted RNA for MCP-1, TNF-α, and IL-8 with glyceraldehyde-3-phosphate dehydrogenase utilized as a housekeeping gene. RT-PCR blots shown are representative of one of three experiments with similar results. ELISA for MCP-1, TNF-α, and IL-8 were performed on cell supernatants. Results shown are mean ± S.E. of at least three experiments. *, p < 0.001 NS versus LPS; #, p < 0.05 compared with LPS; **, p < 0.01 compared with LPS.

FIG. 3. The JNK inhibitor SP600125 decreases LPS-induced MCP-1 expression. Human neutrophils were incubated with SP600125 (2–20 μM), or Me₂SO (0.1%) as diluent control, for 60 min at 37 °C followed by stimulation with LPS (100 ng/ml) for 4 h. RT-PCR was performed on extracted RNA for MCP-1, TNF-α, and IL-8 with glyceraldehyde-3-phosphate dehydrogenase utilized as a housekeeping gene. RT-PCR blots shown are representative of one of three experiments with similar results. ELISA for MCP-1, TNF-α, and IL-8 were performed on cell supernatants. Results shown are mean ± S.E. of at least three experiments. *, p < 0.001 NS versus LPS; #, p < 0.05 compared with LPS; **, p < 0.01 compared with LPS.

FIG. 4. Syk and PI3K activity increases in LPS stimulated neutrophils. A, human neutrophils were incubated for 60 min at 37 °C and stimulated with LPS (100 ng/ml) for the times shown. Syk was immunoprecipitated from cell lysates followed by assessment of Syk autophosphorylation by an in vitro kinase assay. Proteins were separated on SDS-PAGE gels, transferred to nitrocellulose, with radiolabeled bands identified by autoradiography (upper panel). Immunoblotting for Syk was then performed to ensure that equal amounts of Syk were immunoprecipitated from each sample (lower panel). Blots shown represent one of three experiments with similar results. B, human neutrophils were incubated at 37 °C for 60 min and then were stimulated with LPS (100 ng/ml) for the indicated times. Cells were then lysed in boiling Laemmli sample buffer with proteins separated by SDS-PAGE followed by immunoblotting for phospho-Syk (Tyr352). C, isolated neutrophils were stimulated with LPS (100 ng/ml) for the indicated time points or were left unstimulated. PI3K activity was then performed to ensure that equal amounts of PI3K were immunoprecipitated from each sample (lower panel). Blots shown represent one of three experiments with similar results. *

increased within 5 min after exposure to LPS with a return to baseline by 10 min after LPS stimulation. The LPS-induced activity of Syk was confirmed (Fig. 4B) by assessing Syk phosphorylation in lysates, in which full-length Syk was identified, by immunoblotting with a phospho-Syk (Tyr352)-specific antibody.

We next investigated if LPS induces PI3K activity in neutrophils. Neutrophils were stimulated with LPS followed by the immunoprecipitation of p85 and an in vitro lipid kinase assay utilizing phosphatidylinositol as substrate. As seen in Fig. 4C, PI3K is rapidly activated after LPS stimulation in neutrophils with increased activity seen as early as 2 min after LPS stimulation, peak activation at 5 min, followed by a gradual decline in activation back to baseline over the next 35 min. Comparison of Figs. 4A and 4C suggests that activation of PI3K proceeds Syk activation.

Inhibition of Syk or PI3 Kinase Diminishes LPS-induced JNK Activation—To determine if Syk or PI3K were components of the pathway leading to JNK activation, neutrophils
FIG. 5. Inhibition of Syk with piceatannol and PI3K with wortmannin decreases LPS-induced JNK activation without affecting p38 activation. Neutrophils were incubated with piceatannol (10 μM) (A) or wortmannin (50 nM) (B), with Me2SO (0.1%) as diluent control, for 60 min at 37 °C. Neutrophils were then stimulated with LPS (100 ng/ml) for 15 or 30 min or were left unstimulated, followed by cell lysis, and immunoprecipitation for JNK-1. An in vitro kinase assay for JNK activity was performed using c-Jun1–79 as an exogenous substrate (upper panel). Membranes were probed to show that equal amounts of JNK-1 were immunoprecipitated (lower panel). The representative autoradiograph and immunoblot of JNK-1 are from one of three experiments with similar results. Quantification of phosphorylated c-Jun band intensity was performed by PhosphorImager analysis. Activity of c-Jun after LPS stimulation alone was assigned an intensity of 100% with intensity after piceatannol or wortmannin pretreatment normalized to LPS stimulation alone. Results shown are mean ± S.E. of three separate experiments. *, p < 0.001 by one-way ANOVA. C, isolated neutrophils were incubated with or without piceatannol (left) or wortmannin (50 nM) (right), with Me2SO (0.1%) as diluent control, for 60 min at 37 °C. Cells were then stimulated with LPS (100 ng/ml) for 30 min followed by immunoprecipitation for p38 and assessment of p38 kinase activity utilizing the recombinant ATF fragment ATF1,110. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, with radiolabeled proteins detected by autoradiography. Autoradiographs of one of three experiments with similar results are shown. D, neutrophils were incubated under non-suspended conditions for 60 min and then stimulated with LPS (100 ng/ml) (left) or incubated with piceatannol or Me2SO (0.1%) as diluent control) for 60 min then stimulated with LPS (100 ng/ml) (right). Immunoprecipitation for Lyn or pTyr was performed as indicated, proteins were separated by SDS-PAGE, followed by assessment of Lyn autophosphorylation from Lyn immunoprecipitations by an in vitro kinase assay (as per Syk autophosphorylation in Fig. 4) or immunoblotting for Hck for pTyr immunoprecipitations, as indicated. Immunoblotting for Lyn was performed on Lyn immunoprecipitations to ensure that equal amounts of Lyn were immunoprecipitated. Autoradiographs and immunoblots of one of three experiments with similar results are shown.
were incubated with piceatannol (10 μM) to inhibit Syk or with wortmannin (50 nM) to inhibit PI3K prior to stimulation with LPS. Preincubation with piceatannol led to a significant, but incomplete, decrease in LPS-induced JNK activation (Fig. 5A) at 15 and at 30 min, whereas wortmannin inhibited the activation of JNK only at 15 min after LPS stimulation (Figs. 5B). However, inhibition of LPS-induced JNK activation 15 min after LPS stimulation was more pronounced with PI3K inhibition compared with Syk inhibition. To assess if Syk and/or PI3K were involved in other LPS-induced signaling pathways leading to MAPK activation, or were specific to the JNK pathway, we assessed if Syk and/or PI3K were involved in the LPS-induced activation of p38 MAPK, because only p38 MAPK, but not ERK, has been shown to be activated in LPS-stimulated neutrophils. As can be seen in Fig. 5C, neither piceatannol nor wortmannin altered LPS-stimulated p38 activation.

To confirm the specificity of piceatannol inhibition of Syk in our model, we examined the effect of piceatannol on LPS-induced Lyn and Hck activation. Exposure of non-suspended neutrophils to LPS increased Lyn autophosphorylation, as assessed by autophosphorylation of immunoprecipitated Lyn, and Hck phosphorylation, which peaked 5 and 15 min after LPS exposure, respectively (Fig. 5D, left). Incubation with piceatannol prior to LPS exposure did not diminish LPS-induced Lyn autophosphorylation or Hck phosphorylation in non-suspended neutrophils (Fig. 5D, right).

Inhibition of Either Syk or PI3 Kinase Decreases LPS-induced Expression of MCP-1—Because inhibition of Syk and PI3K led to a decrease in JNK activation, and inhibition of JNK decreased MCP-1 expression, we hypothesized that inhibition of Syk and PI3K would also decrease MCP-1 expression. Neutrophils pretreated with piceatannol showed a decrease of −50% in the secretion of MCP-1 protein after LPS stimulation, whereas those pretreated with wortmannin showed a near complete inhibition of LPS-induced MCP-1 expression (Fig. 6A). To determine if transcriptional or translational mechanisms were responsible for the decrease in LPS-induced MCP-1 protein expression with Syk or PI3K inhibition, we assessed the expression of MCP-1 mRNA in LPS-stimulated neutrophils pretreated with piceatannol or wortmannin. As can be seen in Fig. 6, pretreatment with piceatannol prior to LPS stimulation reduced the expression of MCP-1 mRNA by −50% compared with LPS stimulation alone, whereas pretreatment with wortmannin completely inhibited the LPS-induced increase in MCP-1 expression, thereby suggesting a pre-translational mechanism for the decrease in MCP-1 protein expression with Syk or PI3K inhibition.

SP600125 Does Not Inhibit LPS-induced Syk Phosphorylation—In these experiments we utilized concentrations of SP600125 previously shown to be specific for JNK (40). To assist in confirming that our results of a decrease in LPS-induced MCP-1 expression with SP600125 were the result of JNK inhibition and not the result of SP600125 inhibiting Syk, we assessed LPS-induced Syk phosphorylation in the presence of SP600125. As seen in Fig. 7, pretreatment with SP600125 did not affect LPS-induced Syk phosphorylation at any concentration of SP600125 utilized.

PI3 Kinase Is Upstream of Syk—Our findings of a more rapid activation of PI3K compared with Syk after LPS stimulation (Fig. 4) combined with the more significant decrease in LPS-induced JNK activation and MCP-1 expression with PI3K inhibition, compared with Syk inhibition, suggested to us that PI3K may reside upstream of Syk in LPS-stimulated neutrophils. To investigate this possibility, neutrophils were preincubated with wortmannin and LPS-induced Syk autophosphorylation was assessed. The inhibition of PI3K with wortmannin attenuated Syk activation after LPS exposure (Fig. 8). This suggests that PI3K is upstream of Syk in the LPS-induced pathway leading to JNK activation in neutrophils, and that PI3K also activates a Syk-independent pathway leading to JNK.

Syk Associates with TLR4 in Neutrophils after LPS Exposure—To strengthen our findings of the role of Syk in LPS-induced signaling pathways in neutrophils, we investigated the possibility that Syk is a member of the TLR4 signaling complex. Recently, associations of PI3K and Rac1 with TLR2 (36); CXCR4, CD11b, HSP70, and HSP90 with TLR4 (61, 61); and Bruton’s tyrosine kinase with TLR4 (63) have been described. To investigate this possibility, neutrophils were stimulated with LPS, TLR4 was immunoprecipitated from cell lysates, and the presence of Syk was assessed by immunoblotting. As can be seen in Fig. 9, Syk co-precipitates with TLR4 at baseline, but the association increases within 5 min after LPS stimulation, peaks at 10 min, and returns to baseline by 20 min. The kinetics of the increased association of Syk with TLR4 after LPS stimulation correlates with the increase in Syk activity.

Fig. 6. Inhibition of Syk and PI3K decreases MCP-1 protein and mRNA expression after LPS stimulation. Human neutrophils were incubated with piceatannol (10 μM) (A) or wortmannin (50 nM) (B), with Me2SO (0.1%) as diluent control, for 60 min at 37 °C followed by stimulation with LPS (100 ng/ml) for 4 h or were left unstimulated. MCP-1 protein was measured from cell supernatants by ELISA. Results shown are mean ± S.E. of five separate experiments. *, p < 0.05 vs. LPS; #, p < 0.05 LPS versus LPS/piceatannol and LPS versus LPS/wortmannin by one way ANOVA. RT-PCR for MCP-1 was performed with glyceraldehyde-3-phosphate dehydrogenase utilized as a housekeeping gene. PCR products were resolved on 1% agarose gels followed by staining with ethidium bromide. Gels shown are representative of one of five separate experiments with similar results.

Fig. 7. SP600125 does not affect LPS-induced Syk phosphorylation. Isolated non-suspended neutrophils were incubated with SP600125 (5–20 μM) for 60 min at 37 °C then stimulated with LPS (100 ng/ml) for 5 min. Cells were then lysed in boiling Laemmli sample buffer with proteins separated on SDS-PAGE gels followed by immunoblotting for phospho-Syk (Tyr-352). The immunoblot shown is representative of one of three experiments with similar results.
shown previously (Fig. 4, A and B). To confirm that equivalent amounts of Syk were immunoprecipitated, A, representative autoradiograph (upper) and Syk immunoblot (lower) of one of three experiments with similar results. B, quantification of Syk phosphorylation by PhosphorImager analysis. Intensity of Syk phosphorylation after LPS stimulation was assigned 100% with intensity after wortmannin pre-treatment normalized to LPS stimulation alone. Results shown are mean ± S.E. of three separate experiments. *, p < 0.005 by Student’s paired t test.

**FIG. 8.** LPS stimulated Syk activation is PI3K dependent in neutrophils. Neutrophils were isolated and incubated with wortmannin (50 nM), or with Me2SO (0.1%) as diluent control, for 60 min at 37°C and stimulated with LPS (100 ng/ml) for 5 min. Syk was immunoprecipitated from cells followed by performance of an in vitro kinase assay to assess Syk autophosphorylation. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, with radiolabeled bands determined by autoradiography and quantified by PhosphorImager analysis. Immunoblotting for Syk was performed to show that equal amounts of Syk were immunoprecipitated. A, representative autoradiograph (upper) and Syk immunoblot (lower) of one of three experiments with similar results. B, quantification of Syk phosphorylation by PhosphorImager analysis. Intensity of Syk phosphorylation after LPS stimulation was assigned 100% with intensity after wortmannin pre-treatment normalized to LPS stimulation alone. Results shown are mean ± S.E. of three separate experiments. *, p < 0.005 by Student’s paired t test.

**DISCUSSION**

We have identified a novel signaling pathway in LPS-stimulated neutrophils inducing the activation of the MAPK JNK. Although the activation of JNK after LPS stimulation has been described in macrophages (48, 64), to our knowledge this is the first description of the activation of JNK in neutrophils stimulated with LPS. LPS-stimulated JNK activation is not observed in human neutrophils in suspension. The activation of JNK after LPS stimulation is only seen in neutrophils under non-suspended conditions, as was the case in analyzing the activation of JNK after TNF-α stimulation (37).

The focus of this work was to examine the upstream signaling events leading to the LPS-induced activation of JNK and the downstream consequences thereof. In this study we have identified Syk and PI3K as two new components of LPS signaling and demonstrated that both Syk and PI3K participate in JNK activation. The activation of Syk has been shown to occur in neutrophils upon initiation of phagocytosis (65) and after TNF-α stimulation (37), and PI3K activation has been shown in neutrophils upon exposure to IL-8, leukotriene B4, and formylmethionylleucylphenylalanine (58–60); however, the activation of Syk or PI3K after LPS stimulation has not been previously described. In addition, although Syk has been shown to play a role in JNK activation in T cells (66) and in neutrophils...
after TNF-α stimulation (37); and PI3K has been shown to be important in JNK activation in other cell systems after shear stress (26), Fc receptor cross-linking (34) or upon exposure to platelet-derived growth factor (32), angiotensin II (67), or hydrogen peroxide (33), only limited information is available regarding the role of Syk and/or PI3K in LPS-induced JNK activation (48). Collectively, our data show a functional link between Syk, PI3K, and JNK in LPS-stimulated neutrophils not seen in other cell systems.

We have shown a relationship between Syk and PI3K activation in the pathway leading to LPS-induced JNK activation in neutrophils. Although PI3K has been shown to reside downstream of Syk in neutrophils after phagocytosis of IgG-coated erythrocytes (65), no role of JNK was determined. In contrast to those findings, our results suggest that PI3K is upstream of Syk. Not only is PI3K activation induced more rapidly than Syk, but also pretreatment with wortmannin inhibited the increased activation of Syk found after LPS exposure (Fig. 8). In addition our results suggest that neither Syk nor PI3K are involved in signaling pathways leading to p38 activation after LPS stimulation in neutrophils (Fig. 5C), and because we have not been able to detect the activation of ERK MAPK after LPS stimulation in suspended or non-suspended neutrophils, this suggests that both Syk and PI3K are specific to the activation of JNK MAPK in neutrophils after stimulation by LPS.

Our findings further extend the repertoire of neutrophil signaling after exposure to LPS and suggest that up-regulation of genes dependent on JNK increase in non-suspended neutrophils. We show that the activation of JNK led to an increase in the expression and release of MCP-1, as assessed by a decrease in MCP-1 expression with pretreatment with the JNK inhibitor SP600125. Additionally, because pretreatment with piceatannol and wortmannin also decreased LPS-induced MCP-1 expression, this further implicates Syk and PI3K in the pathway leading to JNK activation after LPS stimulation in neutrophils. In contrast, our data suggest that JNK activation is much less important in the LPS-induced regulation of TNF-α and IL-8 mRNA expression, because inhibition of their expression after LPS stimulation only occurred with concentrations of the JNK inhibitor SP600125 previously shown to be less specific for JNK (40).

The use of chemical inhibitors for Syk and PI3K in examining signaling pathways in human neutrophils is unavoidable due to the inability to reliably transfact neutrophils. In an attempt to overcome limitations of inhibitor use, we have utilized concentrations of piceatannol or wortmannin, which have been shown to be specific for Syk or PI3K respectively (37, 65, 68, 69). In particular, although piceatannol inhibits several tyrosine kinases, concentrations of piceatannol of ≤10 μM have been shown to only inhibit the activation of Syk without inhibition of Lyn, Hck, or Fgr activation (65). To further confirm these previous findings in our model system, we have shown that piceatannol does not inhibit LPS-induced Lyn or Hck activation in non-suspended neutrophils (Fig. 5D). Therefore, piceatannol is relatively specific for Syk in this system. Despite these limitations, the use of piceatannol and wortmannin provides strong evidence to suggest that Syk and PI3K are involved in the LPS-induced signaling pathway leading to JNK activation. Likewise for the JNK inhibitor SP600125, we attempted to use concentrations that are specific for JNK. Although difficult to discern intracellularly, the IC_{50} of each JNK isoform in vitro for SP600125 is <0.1 μM, whereas the IC_{50} for most other proteins tested is >10 μM (40). In our series of experiments we carefully utilized concentrations of SP600125 < 10 μM in an attempt to exclude the potential inhibition of non-JNK proteins confounding our results. In addition, to confirm the specificity of SP600125 in our system we have shown that SP600125 did not affect Syk phosphorylation after exposure to LPS. Taken together, our findings of an inhibition of LPS-induced JNK activation, as well as MCP-1 expression, with concentrations of SP600125 of 2 μM support the specificity of our findings of the role of JNK in LPS-induced MCP-1 expression. Finally, in further support of these inhibitor studies is the close correlation between inhibition of each component and the inhibition of MCP-1.

Although both Syk and PI3K regulate JNK activity in LPS-stimulated neutrophils, other pathways undoubtedly exist. This is evidenced by the incomplete inhibition of LPS-induced JNK activation by inhibition of Syk or PI3K (Fig. 5, A and B). The activation of JNK proceeds through the activation of MKK4 and MKK7 (70–73). Although the specific MKK (i.e. MKK4 and/or MKK7) utilized in LPS-induced activation of JNK in neutrophils is unknown, and is the subject of current studies, the possibility exists that the Syk- and PI3K-dependent and -independent pathways may preferentially utilize different MKKs leading to JNK activation after LPS stimulation (70).

We show that the protein-tyrosine kinase Syk is not only activated after LPS stimulation but is associated with TLR4 in quiescent neutrophils and can increase its association with TLR4 after LPS stimulation. This interaction between Syk and TLR4 seen in neutrophils at baseline and upon LPS stimulation may be direct or indirect. Previously Syk has been shown to associate with CD18 and the Fcy receptor, both of which interact with TLR4 upon LPS stimulation in macrophages, which suggests an indirect association of Syk with TLR4 (61, 74, 75). In support of a direct association of Syk with TLR4, TLR4 contains a motif in the intracytoplasmic portion that is a putative Syk-SH2 domain recognition site, suggesting that Syk may associate with TLR4 directly through this domain (76). The elucidation of the mechanisms by which TLR4 and Syk associate in LPS-stimulated neutrophils requires further investigation.

The activation of JNK after LPS stimulation only in non-suspended neutrophils suggests the potential involvement of the integrins CD11b and CD18 in this pathway. Two points strengthen the possibility that CD11b regulates/modulates this pathway. First, we have recently shown the involvement of CD11b in TNF-α-induced JNK activation in adherent neutrophils (37), and second, CD11b has been recently shown to increase association with TLR4, and with CD14, in LPS-stimulated macrophages suggesting that a similar process may occur in neutrophils (61). We are currently examining the potential role of CD11b in the LPS-induced activation of JNK in neutrophils.

Although here implicated in the response of neutrophils to LPS, the role of JNK in the inflammatory response in vivo is unknown. JNK activation was shown to increase in an LPS-induced model of acute lung injury, which was inhibited by the protein-tyrosine kinase inhibitor genistein (77). Markers of acute lung injury were also decreased in this model. In addition, MCP-1 is responsible for the recruitment of monocytes to sites of infection to further the inflammatory response (78, 79). We have shown that Syk activation is much less important in the LPS-induced regulation of TNF-α and IL-8 mRNA expression, because inhibition of their expression after LPS stimulation only occurred with concentrations of the JNK inhibitor SP600125 previously shown to be less specific for JNK (40).
Lipopolysaccharide-induced c-Jun NH\(_2\)-terminal Kinase Activation in Human Neutrophils: ROLE OF PHOSPHATIDYLINOSITOL 3-KINASE AND Syk-MEDIATED PATHWAYS

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