The Phosphatidylinositol 3-Kinase/Protein Kinase B Signaling Pathway Is Activated by Lipoteichoic Acid and Plays a Role in Kupffer Cell Production of Interleukin-6 (IL-6) and IL-10

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Sepsis caused by gram-positive bacteria lacking lipopolysaccharide (LPS) has become a major and increasing cause of mortality in intensive-care units. We have recently demonstrated that the gram-positive-specific bacterial cell wall component lipoteichoic acid (LTA) stimulates the release of the proinflammatory cytokines in Kupffer cells in culture. In the present study, we have started to assess the signal transduction events by which LTA induces the production of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and the anti-inflammatory cytokine IL-10 in rat Kupffer cells. LTA was found to trigger phosphorylation of mitogen-activated protein kinases (MAPK) (p38 MAPK and ERK 1/2) and protein kinase B (PKB). Compared to LPS, LTA was more potent in inducing PKB phosphorylation after 40 min, although we found that the cytokine responses were similar. For both bacterial molecules, blocking phosphatidylinositol 3-kinase 3-kine (PI3-K; LY294002) or Janus kinase 2 (JAK-2; AG490) particularly affected the induction of IL-6 and IL-10 release, whereas TNF-α levels were strongly reduced by inhibition of Src family tyrosine kinases (PP2). All three cytokines were reduced by inhibition of p38 MAPK (SB202190) or the broad-range tyrosine kinase inhibitor genistein, whereas IL-6 release was particularly blocked by inhibition of ERK 1/2 (PD98059). Divergences in the regulatory pathways controlling TNF-α, IL-10, and IL-6 production in Kupffer cells following LPS or LTA stimulation may create a basis for understanding how the balance between pro- and anti-inflammatory cytokines is regulated in the liver following infections by gram-positive or gram-negative bacteria.

In severe sepsis with multiple-organ dysfunction syndrome, the immune defense system intended to protect the host against infection is out of control, and the consequences are severe. Each year, more than a million intensive-care patients die from sepsis in the Western world. Tight control of and balance in the production of inflammatory versus anti-inflammatory mediators are thought to be essential for maintaining immune function and for avoiding damage to vital organs (14, 31, 45). Hence, developing a detailed understanding of the intracellular mechanisms in charge of balancing cytokine levels may be important for obtaining control of deleterious systemic inflammation, preventing the development of multiple-organ dysfunction syndrome, and thereby reducing mortality.

The liver participates in host defense and tissue repair through hepatic cell-cell cross talk that controls coagulation and is vital for the inflammatory processes. When this control is not adequate, a secondary hepatic dysfunction may occur (reviewed in reference 10). Kupffer cells are the liver macrophages, representing by far the largest tissue macrophage population. Activated Kupffer cells may cause injury to hepatocytes by the release of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), or by inducing neutrophil infiltration (3, 20, 27, 55). Located directly in the bloodstream in the narrow liver sinusoids, Kupffer cells may, under some circumstances, release vast amounts of proinflammatory mediators into the circulation. When this action is not quenched by anti-inflammatory counterparts, it may lead to systemic inflammation, sepsis, and damage to several vital organs. In particular, Kupffer cells have been demonstrated to be major producers of circulating interleukin-6 (IL-6) during trauma (43). Moreover, production of the anti-inflammatory cytokine IL-10 by Kupffer cells was recently shown to be essential for protection against peritonitis (13), and IL-10 production and signaling have been proven to be important for protection against liver injury in mice (11, 25). It was also recently reported that an early rise in IL-6 and IL-10 occurred in plasma during liver surgery, whereas the level of the proinflammatory cytokine TNF-α remained low (21). In a bacterial infection, however, Kupffer cells produce significant amounts of TNF-α (4, 51, 55, 56). In infections by gram-negative organisms, lipopolysaccharide (LPS) is the main initiator of inflammatory reactions that may lead to circulatory failure and organ injury. However, gram-positive pathogens that lack LPS have, in the last decades, more often been reported to cause sepsis (6, 34, 47). It has been shown recently that the cell wall component lipoteichoic acid (LTA), unique to gram-positive bacteria, is a potent inducer of cytokine production in Kupffer cells, whereas peptidoglycan is less potent (44). Although LTA, a negatively charged glycolipid anchored to the cell membrane (16), is a molecule with similarities to LPS, the biological activity of the molecule has been mapped to a preserved D-alanine located in the polyglycerophosphate backbone and not to the lipid part.
as in LPS (38). However, the mechanisms by which LTA is recognized by Kupffer cells and the inflammatory signaling pathways that are elicited remain to be examined.

It is clear that Toll-like receptor (TLR) signaling is essential for activation of macrophages and subsequent initiation of the septic response (reviewed in reference 33). The bacterial cell wall components peptidoglycan and LTA, as well as some fungal species, appear to signal primarily through TLR2 and an additional TLR partner (TLR1 or TLR6) (32, 41, 48, 54). The mechanisms of TLR signaling have been subject to extensive study recently, and a TLR signaling pathway starting with formation of a large membrane complex and culminating in the activation and nuclear translocation of nuclear factor kappa B (NF-κB), leading to the activation of a large array of cytokine genes serving pro- and anti-inflammatory purposes, has been revealed in detail (reviewed in references 19 and 59).

Our understanding of this rather non specific mechanism of cytokine gene induction has been expanded by the identification of the involvement of more signaling kinases, like p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2, and phosphatidylinositol 3-kinase (PI3-K), as well as tyrosine kinases (2), but much study remains to be done (reviewed in reference 36).

In the present work, we aimed to dissect some of the signaling events activated in primary Kupffer cell cultures by LTA and LPS and hypothesized that there would be some specificity in the intracellular signaling of LTA and LPS, as well as specificity in the mechanisms controlling cellular secretion of TNF-α, IL-10, and IL-6.

MATERIALS AND METHODS

Bacterial products. Highly purified and active LTA (>99% pure) from Staphylococcus aureus was isolated using a novel isolation procedure (37). The LTA used in our experiments contained <6 pg of LPS/mg of LTA (29). The LPS was derived from Escherichia coli O28:B6 (Difco Laboratories, Detroit, Mich.). All bacterial products were diluted in Laminus amebocyte lysate reagent water (Bio-Whittaker Europe, Verviers, Belgium).

Isolation of Kupffer cells. Kupffer cells were isolated from rat liver (adult Sprague-Dawley rats weighing 300 to 500 g; Institute of National Health, Oslo, Norway) as previously described (44). In brief, Kupffer cell isolation was based on digestion of the liver in the presence of collagenase P (0.24 mg/ml; Roche, Mannheim, Germany), gradient centrifugation in Percoll (Pharmacia Fine Chemicals, Upsala, Sweden), and selective plating (50). The Kupffer cells were plated in 96-well plates (0.2 × 10⁶ cells/well) for cytokine assays or in 10-cm² culture dishes (20 × 10⁶ cells/dish) for protein extracts. The Kupffer cells were then cultured for 48 h in RPMI 1640 (BioWhittaker Europe) in the presence of 10% fetal calf serum (GIBCO, Grand Island, N.Y.), i-glutamine (2 mM; GIBCO), and antibiotics (penicillin and streptomycin; GIBCO) before experiments were performed.

Kupffer cell experiments. Kupffer cells were exposed to bacterial components for various periods of time, as indicated in the figure legends. In some experiments, specific signal transduction inhibitors of p38 MAPK (SB202190; 25 μM), ERK 1/2 (PD98059; 25 μM), PI3-K (Ly294002; 25 μM), protein kinase A (KT5720; 1 μM), tyrosine kinases (genistein; 50 μM), Src family tyrosine kinases (PP2; 5 μM), and JAK-2 tyrosine kinase (AG 490; 25 μM) (all inhibitors were added in a dilution of 1:1,000. The membranes were washed in 5 ml of cold phosphate-buffered saline and then scraped into 250 μl of a buffer containing 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 10 mM CHAPS (3-[3-(cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Sigma, St. Louis, Mo.) and Complete protease inhibitor mix (1 μl/tablet; 10 mg/ml; Roche Molecular Biochemicals, Indianapolis, Ind.). The cell suspensions were sonicated three times for 10 s each time on ice and centrifuged for 5 min at 12,000 × g. Protein samples were then separated on a one-dimensional sodium dodecyl sulfate-polyacrylamide gel (4.0% stacking gel; 10% separating gel; Bio-Rad). Ten micrograms of total protein was loaded in each lane, subjected to electrophoresis, and subsequently transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham). Finally, the results were visualized by autoradiography and quantitated using Totallab image analysis software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, United Kingdom).

Statistical analyses. Data were analyzed in GraphPad Prism version 3.0 by one-way analysis of variance with Tukey’s multiple-comparison test. Differences with P values of ≤0.05 were considered significant.

RESULTS

LTA induces release of TNF-α, IL-6, and IL-10 in primary cultures of rat Kupffer cells. The cytokine release induced in primary cultures of rat Kupffer cells by LTA in comparison with that induced by LPS was studied by ELISA. We found that highly purified LTA (10 μg/ml) induced release of TNF-α (800 pg/ml) (Fig. 1A) 6 h after stimulation and release of IL-6 (4,500 pg/ml) (Fig. 1B) and IL-10 (2,300 pg/ml) (Fig. 1C) 18 h after stimulation, representing at least 60% of the induction

![Graph](http://iai.asm.org/Downloaded from http://iai.asm.org)
obtained by 1 μg of LPS/ml. The time points chosen for analyses of TNF-α and IL-6 were previously optimized (44). IL-10 was also elevated at 6 h (data not shown).

**LTA-mediated cytokine induction by Kupffer cells is not affected by PMB.** In order to show that the stimulatory capacity of LTA was not due to stimulation by LPS, control experiments were performed by adding the specific LPS-inhibiting oligopeptide PMB (10 μg/ml) to LTA and LPS (control) prior to stimulation (Fig. 2). For all cytokines, LPS-mediated stimulation was abolished in the presence of PMB, whereas LTA-mediated stimulation was not significantly affected. This shows that cytokine induction by LTA is not due to contamination by LPS.

**LTA induces phosphorylation of MAPKs and PKB in Kupffer cells.** In order to examine the signaling pathways induced by LTA and LPS in rat Kupffer cells, we performed Western blot analyses of the phosphorylation statuses of three serine-threonine signaling kinases previously shown to be implicated in the inflammatory response (p38 MAPK, ERK1/2, and PKB). Samples were harvested 20 min following stimulation, because that time point was expected to be suitable for detecting phosphorylation of all three kinases simultaneously. This is primarily due to the fact that p38 MAPK has been reported to be downregulated 30 min after activation in mouse Kupffer cells (26). In Fig. 3A, the kinase phosphorylation pattern for one representative experiment is shown, and it indicates that phosphorylation of all three kinases was induced following 20 min of incubation with LTA (10 μg/ml) or LPS (1 μg/ml). Compared to LPS, LTA tended to induce a similar degree of phosphorylation of p38 MAPK but less phosphorylation of ERK 1/2 after 20 min. In contrast, PKB phosphorylation appeared to be slightly higher in cells following exposure to LTA than after exposure to LPS. It should be noted that there is a reverse correlation between amounts of total ERK and phosphorylated ERK detected in this and all experiments performed. This is not due to inaccurate loading, since no corresponding variations were found in the amounts of total PKB or p38 MAPK on the same blot. We next performed time course analyses of ERK 1/2 (Fig. 3B) and PKB (Fig. 3C) phosphorylation, showing the relative induction of kinase phosphorylation for three independent experiments. We found that phosphorylation of both kinases was further increased after 40 min of stimulation, and we could observe a significantly higher induction of PKB phosphorylation after 40 min of stimulation by LTA than after stimulation by LPS. There was also a tendency (nonsignificant) for a higher level of ERK 1/2 phosphorylation in cells stimulated by LPS for 20 min. The quantification data in Fig. 3B are not adjusted for total ERK, due to the so-far-unexplained downregulation mentioned above. These observations indicate that LTA is a stronger activator of PKB than is LPS.

**PKB and ERK are involved differently in TNF-α, IL-6, and IL-10 production.** To further examine the signaling pathways involved in cytokine induction by LTA, we added specific inhibitors of ERK1/2 (PD98059; 25 μM), p38 MAPK (SB202190; 25 μM), PI3-K (signals upstream of PKB) (Ly294002; 25 μM), or cyclic-AMP-dependent protein kinase/PKA (KT5720; 1 μM) to Kupffer cell cultures prior to the addition of LTA (10 μg/ml) or LPS (1 μg/ml) and examined the release of TNF-α, IL-6, and IL-10 (Fig. 4). Concentrations of inhibitors were chosen according to 50% inhibitory concentration (IC50) values and specificities reported by others (9, 12, 18, 42). Cell culture viability, as assayed by trypan blue staining, was >80% at harvesting time for all cultures incubated with the inhibitor concentrations used, showing that the inhibitors had no general toxic effect on the cells (data not shown).

Total inhibition of ERK1/2, p38 MAPK, and PKB phosphorylation by their respective inhibitors 20 min after the addition of LTA was confirmed on Western blots (data not shown). For TNF-α (Fig. 4A), inhibition of p38 MAPK and PKA had the most profound effects, reducing TNF-α levels by 75%, although none of the inhibitors were able to totally block TNF-α release. The release of IL-6 was reduced by all inhibitors (Fig. 4B) but was primarily blocked by interruption of ERK 1/2. IL-10 levels (Fig. 4C) were particularly reduced by inhibition of p38 MAPK, PI3-K, and PKA (>50% reduction) and less in the presence of ERK1/2 inhibitor.

Due to the observation that PKB is phosphorylated follow-
ing LTA (and LPS) stimulation, we were specifically interested in the effect of inhibiting the upstream PKB-activating kinase, PI3-K, on cytokine expression. Various concentrations of the PI3-K inhibitor Ly294002 were added to the cell culture prior to stimulation, and TNF-α, IL-10, and IL-6 levels were measured after 6, 6, and 18 h (Fig. 5). We found no difference between the effects on LTA- and LPS-mediated stimulation, but we observed different effects on production of the three cytokines. Whereas TNF-α production was not significantly affected by PI3-K inhibitor levels below 100 μM (at 100 μM, nonspecific inhibitor effects are likely to occur) (Fig. 5A), PI3-K inhibitor levels lower than the IC50 (1.4 μM) were able to attenuate the production of IL-10 in the same sample (Fig. 5B). Somewhat higher levels of Ly294002 (10 μM) were needed to significantly affect IL-6 production after 18 h (Fig. 5C). The effects of inhibitors on LPS- versus LTA-stimulated cultures showed no specific differences.
Src and JAK tyrosine kinases are differently involved in TNF-α, IL-6, and IL-10 production. The involvement of tyrosine kinases in the inflammatory response to bacterial products have been a recent focus for research. We aimed to examine the importance of specific tyrosine kinases on TNF-α, IL-6, and IL-10 production. Two main classes of tyrosine kinases have recently been applied to TLR signaling and cytokine regulation, Src and Janus kinase 2 (JAK-2) (15, 42), and these were the subjects of our next experiment. Inhibitors of Src family kinases (PP2; 5 μM) and JAK-2 (AG 490; 25 μM), as well as the broad-range tyrosine kinase inhibitor genistein (50 μM), were added to the Kupffer cell cultures prior to stimulation by LTA or LPS. Trypan blue staining demonstrated no significant effect of the inhibitors on cell culture viability at the harvesting time point (viability, >80%). Figure 6A demonstrates that TNF-α production is specifically attenuated by inhibition of Src family tyrosine kinases but significantly less by inhibition of JAK-2 for LPS-stimulated samples. For IL-6 (Fig. 6B), the inhibitory pattern was reversed, showing significantly stronger effects of JAK-2 inhibition in both LPS- and LTA-stimulated cultures. Finally, IL-10 production appeared to be reduced by ~30 to 50% in the presence of each of the specific inhibitors, whereas the nonspecific tyrosine kinase inhibitor (genistein) reduced IL-10 levels to those of unstimulated cell cultures, suggesting that more than one class of tyrosine kinases mediates the production of IL-10.

**DISCUSSION**

We demonstrate in this study clear similarities between the responsiveness of primary cultures of Kupffer cells to LTA.
from gram-positive bacteria and LPS from gram-negative bacteria. This is the first report of intracellular signaling pathways involved in LTA-mediated stimulation of the main macrophage population of the body, the Kupffer cells. LTA is a membrane-linked cell wall component unique to gram-positive bacteria and is believed to be a gram-positive equivalent of LPS from gram-negative bacteria (37). In this and a previous report, it was shown that Kupffer cells in the liver react by release of cytokines in response to highly purified *S. aureus* LTA, whereas they show insignificant responses to the main cell wall component, peptidoglycan (44).

In this study, we found that levels of signaling kinase phosphorylation induced by LPS or LTA showed only a few differences. Whereas LPS appeared to be superior to LTA with respect to induction of ERK phosphorylation, LTA induced significantly more phosphorylation of PKB 40 min after stimulation. The same relationship was also observed at 20 min (Fig. 3A). This is supported by another report showing that live *S. aureus* organisms have the ability to induce PKB activation through TLR2 in monocytes (2), and we propose that LTA may be the membrane component responsible for PKB activation. However, although inhibition of the PKB pathway by PI3-K inhibitor influenced the production of IL-10 and IL-6 much more strongly than TNF-α, we did not find that LTA was a stronger inducer of IL-10 or IL-6 in this study.

Although LPS and LTA signal through different TLRs—TLR4 and TLR2/TLR6, respectively (7, 39, 58)—and were found to have profoundly different effects on leukocyte movements and endothelial interactions in vivo (57), our data clearly show that there are no major differences between the intracellular signaling pathways by which LPS and LTA induce TNF-α, IL-6, and IL-10 in Kupffer cells. We performed a PMB test to make sure we were comparing the responses to two very different molecular components, and we conclude that pure *S. aureus* LTA induces strong cytokine responses in Kupffer cells comparable to those induced by *E. coli* LPS.

We further present data showing how production of TNF-α, IL-10, and IL-6 depend on partially different signaling pathways. The mechanisms balancing the levels and time courses of pro-versus anti-inflammatory mediators in tissue macrophages are only beginning to be understood and can best be worked out through comparative studies of various combinations of cytokines produced from the same cell source in the infected tissue. Kupffer cells are important producers of TNF-α, IL-10, and IL-6 in vivo, and the balance in the levels of these cytokines is not only essential for immune responses locally in the liver but may also alter systemic levels of pro- and anti-inflammatory cytokines (11, 13, 25, 43, 56). IL-10 has the ability to suppress proinflammatory cytokine release and may protect against the onset of irreversible sepsis (28), and hence, the initial local TNF-α—IL-10 balance is crucial for further development of the inflammatory process. IL-6 produced by Kupffer cells appears to serve both pro- and anti-inflammatory purposes (27, 43). It should be mentioned that human Kupffer cells in our hands have displayed a reduced ability to produce IL-10 when exposed to LPS, whereas TNF-α production was intact. Others have also made observations showing that patients suffering from hepatitis B had strong upregulation of proinflammatory cytokines without the corresponding production of IL-10 (30). These findings should be examined further.

We aimed, in this study, to search for specificity in the signaling events leading to TNF-α, IL-10, and IL-6 production by Kupffer cells by the use of a number of specific signal transduction inhibitors with the purpose of uncovering potential early control points of the proinflammatory–anti-inflammatory balance. In Fig. 7, our observations are interpreted as a simplified illustration showing how Kupffer cell production of TNF-α, IL-6, and IL-10 depends on the activities of partially different signaling kinases.

The fact that ERK1/2 activity is dispensable for the production of IL-10 in Kupffer cells and monocyte-derived macrophages has been reported by others (23, 46), and quite recently, the importance of p38 MAPK and ERK 1/2 for TNF-α and IL-10 production was examined in primary mouse Kupffer cell cultures stimulated by LPS (23). By using the same inhibitors as in our experiments, the authors found that inhibition of p38 MAPK blocked both TNF-α and IL-10 expression, whereas ERK1/2 inhibition had a strong effect on the production of TNF-α compared to IL-10. In our experiments, we observed no significant reduction in TNF-α levels caused by the ERK 1/2 inhibitor. Others have shown quite limited reduction of TNF-α levels by inhibition of ERK in monocytes activated by live *S. aureus* or LPS (35, 52). The observation that IL-6 production strongly depends on ERK 1/2 activity is strengthened by a previous study of LPS-stimulated Kupffer cells (5).

We found that pretreating cells with a PKA inhibitor (KT5720) decreases the release of all three cytokines, and an inhibitory effect on IL-10 production may be supported by experiments performed with monocytes (49). Studies of the effects of PKA inhibition on TNF-α and IL-6 expression, however, are somewhat conflicting. What has confused researchers...
for more than a decade is the fact that the PKA activator, cyclic AMP, is a strong inhibitor of TNF-α production in what may be a PKA-independent manner (24, 40). PKA itself appears to serve other, so-far-unexplained functions in mononuclear cells. The PKA inhibitor KT5720 is also not as specific as the other inhibitors used and may interfere with signaling downstream of PI3-K (9).

According to our results, the most striking difference between TNF-α and IL-10 production lies in the dependence on PI3-K and JAK-2. Association of PI3-K activation with reduced inflammatory responses has been shown in monocytes and in a murine sepsis model (17, 53), and recently, a role for JAK-2 upstream of PI3-K in LPS signaling was published (42), offering an explanation for our observation that JAK-2, like PI3-K, is essential for IL-6 and IL-10 production but dispensable for the regulation of TNF-α. Interestingly, PI3-K activation is known as the key initial response to insulin (1), and insulin treatment in vivo has been proven to reduce circulating inflammatory cytokines, to induce anti-inflammatory responses has been shown in monocytes and in a murine sepsis model (17, 53), and recently, a role for JAK-2 upstream of PI3-K in LPS signaling was published (42), offering an explanation for our observation that JAK-2, like PI3-K, is essential for IL-6 and IL-10 production but dispensable for the regulation of TNF-α. Interestingly, PI3-K activation is known as the key initial response to insulin (1), and insulin treatment in vivo has been proven to reduce circulating inflammatory cytokines, to induce anti-inflammatory cytokine levels, and to be beneficial in a model of thermally injured rats (8, 22).

The involvement of Src tyrosine kinases in TNF-α production has recently been shown in a murine macrophage cell line (15). In our experiments, we reveal that Src kinase inhibitors have no effect on IL-6 release and only limited effects on IL-10 levels. A slight Src-mediated inhibition of IL-10 may be a secondary response.

This comparative study indicates some of the signaling events activated by LTA in Kupffer cells and uncovers the relative importance of the different intracellular signaling pathways in controlling the balance between TNF-α, IL-10, and IL-6. The local and initial proinflammatory–anti-inflammatory cytokine balance is most probably decisive for the total inflammatory outcome of a bacterial infection, and the optimal balance may thereby limit the release of excess proinflammatory mediators into the organs and the circulation, leading to sepsis and multiorgan damage. Medication inactivating the total tissue macrophage response to bacterial molecules primes for the spread of microorganisms to surrounding tissue and circulation and may subsequently lead to sepsis as well. A fine-tuned control of signaling pathways in tissue macrophages in order to balance pro- and anti-inflammatory responses is a presupposition for a beneficial immune response. Obtaining detailed knowledge of how cytokine production is controlled by tissue macrophages may open the future possibility of preventing local inflammation from running out of control.

In summary, our data support the notion that LTA is a major immunostimulatory component of S. aureus, equivalent to LPS in infections by gram-negative organisms. LTA induces phosphorylation of PKB that is activated through the PI3-K pathway, and an active PI3-K pathway, as well as JAK-2 activity, is involved in the induction of IL-6 and IL-10. We hypothesize that JAK-2 and PI3-K may serve as mediators of the anti-inflammatory response represented by IL-10, whereas a Src family kinase is important for the burst of TNF-α. Future research will reveal if these signaling kinases represent possible drug targets for tuning the proinflammatory–anti-inflammatory cytokine balance and play a role in protection against sepsis.