Activation of the STAT pathway in acute lung injury

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1Pulmonary and Critical Care Division and 2Department of Physiology, Tufts-New England Medical Center, and 3Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111; 4Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510; and 5Department of Pathology, Brown University School of Medicine, Providence, Rhode Island 02912
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Severgnini, Mariano, Satoe Takahashi, Liliana M. Rozo, Robert J. Homer, Charles Kuhn, Jhung W. Jhung, George Perides, Michael Steer, Paul M. Hassoun, Barry L. Fanburg, Brent H. Cochran, and Amy R. Simon. Activation of the STAT pathway in acute lung injury. Am J Physiol Lung Cell Mol Physiol 286: L1282–L1292, 2004. First published January 16, 2004; 10.1152/ajplung.00349.2003.—Acute lung injury (ALI) is a devastating clinical problem with a mortality as high as 60%. It is now appreciated that ALI represents a cytokine excess state that involves the microvasculature of multiple organs. The signal transducers and activators of transcription (STAT) family of transcription factors activate critical mediators of cytokine responses, but there is limited knowledge about their role in mediating ALI. In the present study, we demonstrate that the STAT transcription factors are activated rapidly in the lungs after intraperitoneal and intranasal LPS administration in mice. We also demonstrated that LPS activates both the STAT kinases, Src and JAK, in the lung with kinetics that are consistent with STAT activation. LPS treatment resulted in STAT3 activation throughout the resident lung cells, as well as in the recruited inflammatory cells. Whereas direct LPS treatment did not lead to STAT activation in cultured epithelial or endothelial cells, IL-6 activated STAT3 in both of these cell types. Furthermore, IL-6 was induced by LPS in serum and in the lung with kinetics consistent with STAT3 activation, suggesting that IL-6 may be one mechanism of STAT activation by LPS. In addition, STAT activation required reactive oxygen species, as the overexpression of catalase in mice prevented LPS-mediated STAT activation in the lung. STATs may be a common pathway for mediating ALI regardless of the inciting factor, as STAT activation also occurred in both a gastric acid aspiration and acute pancreatitis model of ALI. Finally, STATs are activated in the lung long before signs of ALI are present, suggesting that the STAT transcription factors may play a role in initiating the inflammatory response seen in the lung.

signal transducers and activators of transcription; lipopolysaccharide; reactive oxygen species

ACUTE LUNG INJURY (ALI), as manifested by the acute respiratory distress syndrome, is a devastating clinical problem with an annual incidence in the United States high as 75 per 100,000 population, and a mortality rate of 40–60% (6, 25). Whereas it is initiated by a variety of insults, both direct (gastric aspiration) and indirect (sepsis), the pathological end point is diffuse alveolar damage. It is now appreciated that ALI represents a cytokine excess state that involves the microvasculature of multiple organs. The outcome of ALI is thought to depend, in part, on the balance between proinflammatory cytokines that promote tissue destruction (tumor necrosis factor-α and IL-1), and anti-inflammatory cytokines that promote healing (IL-10 and IL-13). Whereas lung-protective ventilation strategies have recently been shown to significantly decrease cytokine production and mortality, no such benefit has been seen with immune-modulating agents, such as TNF receptor blockade or IL-1 receptor antagonists (9, 46). This is likely due to the function of cytokines as mediators of innate immunity, as well as inflammation, and that patient selection for clinical trials has not made distinctions between the different inciting mechanisms of ALI. Importantly, these treatment failures point out our lack of understanding about the cellular and molecular mechanisms that underlie ALI.

Downstream signal transduction pathways that have been shown to participate in mediating lung inflammatory responses include the NF-κB, MAPK, and phosphotidylinositol 3-kinase pathways (54). NF-κB has been shown to be upregulated in animal models as well as in humans with ALI (61). However, it is not known whether NF-κB plays a causative role in this disease. The signal transducers and activators of transcription (STAT) family of transcription factors has a similar profile of immune-regulatory and inflammatory genes as NF-κB, but there is limited knowledge about its role in mediating ALI (30).

The STAT family of transcription factors was initially identified as cytokine and growth factor-inducible DNA-binding proteins (18, 27). The STAT proteins exist in a latent form in the cytoplasm and, on receptor activation by cytokines, become phosphorylated on tyrosine residues by members of the Janus kinase (JAK) family (JAK1, JAK2, JAK3, and Tyk2), which are physically associated with the receptor (79). The Src kinase family has also been shown to be important in growth factor-induced tyrosine phosphorylation and, in some instances, for cytokine-induced activation of STATs (14, 42). This phosphorylation is obligatory for STAT activation and mediates dimerization via p-Tyr-SH2 domain interactions, resulting in translocation of the STATs to the nucleus, where they bind to DNA and regulate specific gene expression. In addition, our laboratory (63, 64) has previously demonstrated that the JAK-STAT pathway can be activated by oxidative stress and that growth factor-mediated STAT activation requires reactive oxygen species (ROS) that likely function through the inhibition of phosphatases. STAT target genes include cytokines, adhesion molecules, and inflammatory mediators, such as nitric oxide synthase and LPS binding protein (LBP) (39, 40, 60). Data from

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various STAT knockout mice demonstrate that STATs are absolutely required for cytokine signaling and immune responses (32).

Sepsis is the most common cause of ALI, accounting for >30% of cases in humans, and is the major cause of mortality in these patients (74). Endotoxin or LPS from the wall of gram-negative bacteria has been implicated in pathogenesis of ALI in humans and, when administered to animals, results in similar pathology with neutrophil sequestration and increased vascular permeability in the lung (81). LPS stimulates inflammatory signaling via the toll-like receptor 4 member that is expressed on phagocytic cells as well as nonphagocytic cells [see Dalpke and Heeg for review (17)]. Recent studies have demonstrated that the STAT pathway and its upstream kinases are important for the host innate immune response to LPS, both in vitro and in vivo, making STATs possible contributors to LPS-mediated ALI (37, 41, 68, 84). Here we examine the activation of the STAT pathway in an animal model of ALI induced by LPS. In addition, we evaluate STAT activation in two other animal models of ALI, i.e., acute pancreatitis and gastric aspiration.

**METHODS**

Reagents. TNF-α and IL-6 were purchased from R&D Systems (Minneapolis, MN). LPS from *Escherichia coli* (serotype 055:B5) and all other chemicals, unless indicated, were purchased from Sigma-Aldrich (St. Louis, MO). Pentobarbital sodium, ketamine, and xylazine were purchased from Fort Dodge Animal Health (Fort Dodge, IA). STAT1, STAT3, p44/42 MAPK, phospho-STAT3 (Y705), phospho-STAT1 (Y701), phospho-JAK2 (Y1007/1008), and phospho-Src (Y416) were purchased from Cell Signaling (Beverly, MA). Phospho-p44/42 MAPK antibody was purchased from Santa Cruz. Anti-STAT6 (Y416) were purchased from Cell Signaling (Beverly, MA). Phospho- 

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**Cell culture.** Human microvascular endothelial cells were purchased from Cambrex (Walkersville, MD) and grown in EGM-2 MV SingleQuots media (Cambrex) before being placed in RPMI ( Gibco, Grand Island, NY) media for 24 h for quiescence. MLE-12 cells are a cell line derived from lung tumors of transgenic mice carrying the transcriptional control of the human surfactant protein C promoter. These transformed mouse lung tumor cells have morphological and functional characteristics of alveolar type II cells (78). They were kindly provided by Dr. Heber Nielsen (Tufts University, Boston, MA) and repeatedly passaged for use in the experiments. Cells were plated and grown to confluence in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml β-glutamine. Animals. Male BALB/c or C57BL/6 mice were obtained from either Jackson Laboratory (Bar Harbor, ME) or Taconic Laboratory (Germantown, NY). Mice were kept on a 12:12-h light-dark cycle with free access to food and water. The Tufts University Animal Care and Research Committee approved all experiments. During experiments, all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals.

Experimental protocols for lung injury models. We used well-established and reproducible models of ALI that have been described previously by us and others (50, 71, 83). Male BALB/c mice were injected intraperitoneally (IP) with 6 mg/kg of LPS or with sterile PBS as a control. Animals treated intranasally (IN) were anesthetized with pentobarbital (60 mg/kg) and were killed via en bloc removal of vital organs. Blood was aspirated from the right heart for serum, and the blood was removed from the lungs by injecting ice-cold PBS with 2 mM NaVO₃ and 5 mM NaF through the right heart. The lungs were immediately placed in liquid nitrogen and processed for protein or ELISA, as described below. For the acute pancreatitis model, C57/BL6 mice received hourly IP cerulein (50 μg/kg, 1/h) for 6–8 h before being killed, as previously described (83).

**Histological examination.** Mice were anesthetized with pentobarbital, and median sternotomy was performed. The trachea was cannulated with a 20-G angiocatheter, and the lungs were inflated to 25 cm with neutral buffered 10% formalin and were subsequently paraffin embedded. Slides were baked at 60°C for 30 min, deparaffinized, brought to water, subjected to endogenous peroxidase block with 3% H₂O₂ in methanol for 10 min at room temperature, and rinsed in PBS. Protein block (2% normal goat serum, 1% BSA in 0.1 M Tris, pH 7.0, 0.15 M NaCl) was applied for 20 min at room temperature, drained, and then anti-STAT3pY6 was applied at 1:100 for 90 min. Slides were rinsed in PBS three times for 3 min, and then horseradish peroxidase anti-rabbit was applied (powervation polymerized horse-radish peroxidase anti-rabbit) for 30 min at RT. Slides were rinsed with PBS three times for 3 min and then developed with diaminobenzidine (liquid from Vector Laboratories) for 5 min. Slides were rinsed with water and then counterstained with hematoxylin. The lungs were then sectioned and stained with hematoxylin and eosin.

**Adenoviral infection.** Mice were anesthetized with IP ketamine (90 mg/kg) and xylazine (5 mg/kg) before adenoviral infection. Adenovirus (10⁹ -10¹⁰ particles/mouse in 25 μl total sterile PBS) containing catalase was instilled in a dropwise manner to each nostril on days 1 and 3. Control mice received empty adenovirus [Ad green fluorescent protein (GFP)] IN at the same doses. The replication-deficient adenovirus containing catalase was kindly provided by Dr. John Englehardt (University of Iowa, Des Moines, IA). The adenovirus containing GFP was kindly provided by Dr. Daniel Frantz and Steve Shoelson (Harvard University, Boston, MA). On day 5, mice were treated with LPS IP (6 mg/kg) for the indicated times before being killed.

**Electrophoretic mobility shift assay.** After the indicated treatments, nuclear extracts were obtained as previously described (63). Nuclear extracts (12 μg) were added to 32P-end-labeled oligonucleotide m67 SIS-PDGF-inducible element (SIE) probe (~30,000 counts/min 32P-200 fmol) for 30 min at 30°C. The reaction mixture was electrophoresed through 5% polyacrylamide gels containing 2% glycerol in 0.5× Tris-borate-EDTA buffer at room temperature. The gel was then dried and subjected to autoradiography.

**Western analysis.** Whole lungs were homogenized in lysis buffer, as previously described (63). Lysates (40 μg of protein) were run on an 8% SDS polyacrylamide gel and transferred to nitrocellulose. The membrane was then blotted with the indicated antibody and processed via chemiluminescence (Pierce).

**Serum and lung tissue quantification of TNF-α and IL-6.** Blood was obtained via aspiration after right heart puncture, and serum was prepared by centrifugation. Lung tissue was homogenized with four volumes of PBS and centrifuged at 10,000 g for 20 min at 4°C. The supernatant was used for the determination of TNF-α and IL-6 by ELISA. The levels of TNF-α and IL-6 in the serum and lung tissue were quantitated by ELISA kits purchased from R&D Systems.

**Statistical analysis.** Data are expressed as means ± SE. An ANOVA was performed with Statview statistical analysis software, and a difference was accepted as significant if the P value was ≤0.05, as verified by the Bonferroni-Dunn post hoc test. All experiments were repeated at least three times, and all experiments represent data on three to six mice per data point. Student’s t-test was performed on the means of two sets of sample data and considered significant if the P value was ≤0.05.
RESULTS

STATs are activated in an LPS model of ALI. EMSA and Western blot analysis were used to investigate whether members of the STAT family of transcription factors are activated in an LPS model of lung injury. LPS was administered IP to BALB/c mice, and their lungs were examined for STAT activation at time points ranging from 1 to 24 h. Total lysates were prepared from whole lung and analyzed by EMSA by using the high-affinity c-fos SIE as the probe (75). This probe binds with high affinity to most STATs. LPS treatment resulted in robust STAT DNA binding activity that was maximal at 1 h and still present at 24 h (Fig. 1A). To confirm the identity of these LPS-induced SIE binding complexes, antibody supershift analysis was performed on lysates from mice treated with LPS for 1 h, because this was the maximal point of activation. Addition of anti-STAT3 antiserum to the binding reactions supershifted the upper complex but left the lower complex unaltered (Fig. 1B). Addition of anti-STAT1 antibody made against the unique COOH-terminal peptide of STAT1 supershifted the lower complex and left the upper complex binding DNA. Antibody to STAT6 did not shift the DNA binding complexes (data not shown). Thus LPS treatment induces STAT1 and STAT3 in mouse lung rapidly after LPS treatment.

The phosphorylation of a conserved C-terminal tyrosine is required for STAT activation and translocation to the nucleus. To further confirm the activation of STATs by LPS in mouse lung and to examine in more detail the time course of this activation, we examined the phosphorylation state of STAT1 and STAT3 with anti-pY701 STAT1 and anti-pY705 STAT3 phosphospecific antibodies. LPS treatment resulted in a significant increase in STAT1 and STAT3 tyrosine phosphorylation (Fig. 1C), consistent with the induction of STAT DNA binding activity by EMSA. The doublet seen with the phosphospecific STAT3 antibody represents the two different STAT isoforms, STAT3-α and STAT3-β, which arise from differential splicing, as an antibody to the unique C-terminus of STAT3-α only recognizes the upper band (data not shown) (10). The same blot was stripped and rebotted with a STAT3 antibody that recognizes both isoforms to demonstrate that the increase in STAT phosphorylation was not due to increased protein expression (Fig. 1C), although, at the later time points, there is some upregulation of STAT3. Analysis of the time points from both the EMSA and Western blot analyses indicates that the kinetics of STAT1 and STAT3 activation are very different: STAT1 activation is observed as early as 1 h and is at baseline by 12 h, whereas STAT3 activation occurs as early as 30 min and persists at 24 h. The kinetics of STAT3-α and STAT3-β are also distinct, with STAT3-β activation occurring earlier and lasting for a more prolonged period of time than that seen with STAT3-α. Given that STAT3 has been previously identified as an acute-phase response gene in the liver and involved in cellular responses
JAK and Src kinases are activated in the lung by LPS.

Whereas cytokine activation of STAT tyrosine phosphorylation is largely mediated by the JAK kinase family, some data show that the Src family kinases may also be involved (14). In addition, Src and JAK kinase have been shown by others and us to reside in a common pathway with Src upstream of JAK kinase (12, 64, 76). To determine whether known STAT kinases are being activated by LPS in vivo, in the lung, with kinetics correlating with STAT activation, Western blot analysis was performed by using phosphospecific antibodies to JAK and Src kinases. These antibodies only recognize the activated form of those kinases. As shown in Fig. 2, LPS activates JAK2 and Src kinase in whole lung. The activation of JAK and Src kinases was first observed at 30 min and peaked at 2 h, which correlates with the tyrosine phosphorylation of STAT3 after LPS treatment. At 12 h, there is a downregulation of kinase phosphorylation back to untreated levels of both kinases, followed by a wave of subsequent activation thereafter, which was also similar to that seen with STAT3 activation.

We wanted to compare the timing of LPS-mediated activation of the STAT kinases in the lung to that seen with the MAPK and NF-κB pathways. These pathways interact closely with one another. The Src kinases have been shown to be upstream of Raf and MAPK activation, and MAPKs have been shown to be required for maximal STAT activity by mediating STAT serine phosphorylation (19, 24). LPS-induced MAPK activation, as determined by Western blot analysis with phosphospecific ERK1/ERK2, has kinetics of activation that are nearly identical to JAK and Src kinase activation (Fig. 2). Similar to what others have shown in rats, LPS-induced NF-κB activation peaked at 2 h and started to dissipate after 6 h, as determined by Western blot analysis using antibody to IκB, which gets degraded with NF-κB activation (7). These results suggest that there is almost simultaneous activation of these signaling pathways in the lung by LPS.

STATs are activated in hydrochloric acid and acute pancreatitis models of lung injury. Given that STATs are pivotal in immune and inflammatory responses, it seemed likely that STATs are also activated in models of ALI other than that produced with LPS. To determine whether this was the case, two well-described lung injury models were examined: 1) an acid aspiration model (direct injury); and 2) an acute pancreatitis model (indirect injury) (50, 55). Both of these models are highly relevant to human disease because gastric aspiration and acute pancreatitis are associated with >30% incidence of ALI (31, 66). BALB/c mice were treated with IN HCl (pH = 1.2) for 2 h before being killed. STAT3 activation occurred after HCl treatment, as determined by EMSA (Fig. 3A) and Western blot analysis (data not shown).

To investigate whether STATs are induced in ALI caused by acute pancreatitis, mice were given hourly IP cerulein, a pancreatic secretagogue, for either 6 or 8 h before being killed. As seen in Fig. 3, STAT3 activation occurs at both 6 and 8 h, as determined by Western blot analysis. This activation precedes the lung injury that occurs in this model, which has been shown to be maximal at 12 h (83). Thus, in addition to finding STAT3 activation in an LPS model of lung injury, we demonstrate that it also occurs in two other models of ALI, suggesting that the STAT pathway may be common for mediating ALI, regardless of the inciting stimulus. To characterize STAT activation in ALI in more detail, the remainder of this paper will focus on characterizing STAT activation in the LPS model of ALI.

Localization of STAT activation. LPS has been shown to directly activate STATs in phagocytic cells as well as in nonphagocytic cells (15, 41). The robust and early STAT activation seen on Western blot analysis using whole lung lysates suggests that STAT activation is likely occurring in multiple cell types throughout the lung. To identify where in the lung LPS-mediated STAT3 activation was occurring, and how this activation was changing over time, immunohistochemistry was performed on lung sections of mice treated with LPS from 30 min to 72 h by using a phosphospecific STAT3 antibody. When STATs are activated, they translocate to the nucleus, hence nuclear staining indicates the site of STAT activation. STAT3 immunohistochemistry was performed on lung sections at various times after LPS administration. There
is minimal STAT3 activation in the airway epithelium of PBS-treated control mice, but, after 30 min of LPS treatment, STAT3 activation is markedly increased in the epithelium of the central airways as well as in the endothelium of large veins (Fig. 4 and data not shown). After 2 h of LPS treatment, which showed maximal STAT3 activation by Western blot analysis, there is a marked increase in STAT3 activation throughout the lung, including all of the endothelial beds, the central and distal airway epithelium, parenchymal epithelium, and in smooth muscle found in vessel walls (Fig. 4). By 6 h, the overall staining starts to diminish except in the recruited inflammatory cells (monocytes and neutrophils), which have increased in number as well as have evidence of increased STAT3 activation (data not shown). At 48 h post-LPS treatment, STAT3 activation in the lung is almost back to baseline with the exception of the airway epithelium, which shows some activation (Fig. 4). Interestingly, there also appears to be STAT activation in the cardiac myocytes harvested with the lung specimens, which is consistent with that found by others showing LPS directly activating STAT3 in cardiac myocytes in vitro (15). Thus STAT3 activation is observed first in the airway epithelium and then occurs in multiple endothelial cell beds as well as recruited inflammatory cells. Whereas staining is seen in the parenchymal epithelium, it does not appear as intense as that seen in the airways.

**LPS fails to activate STATs in cultured lung cells.** To determine whether LPS may be directly activating STATs, cell culture experiments were employed utilizing human lung microvascular endothelial cells and MLE-12 cells, type II-like lung epithelial cells, because both of these cell types exhibited STAT activation by immunohistochemistry and are critical sites of lung injury (78). The dose of LPS employed in our experiments has been shown to induce STAT activation as well as to induce downstream gene expression in other cell types (15). Growth factor and cytokine activation of STATs typically occur rapidly (within 10 min); therefore, cells were treated with LPS for 10 min before total lysates for Western blot analysis were obtained by using phosphospecific (Y705) STAT3 antibody. As seen in Fig. 5, LPS does not induce STAT3 activation in either cell type. Similarly, longer time points of LPS treatment (30 and 60 min), as well as a higher dose of LPS (20 µg/ml), did not lead to STAT activation (data not shown). LPS treatment for 24 h did not significantly increase ST3 phosphorylation in MLE-12 cells and resulted in 100% cell death in human lung microvascular endothelial cells (Fig. 5). LPS derived from Shigella bacteria also had no effect on STAT activation (data not shown). Soluble CD14 and LBP in serum enable CD14+ cells, such as endothelial cells, to respond to LPS; therefore, these experiments were repeated with LPS by using 2.5% FCS, which contains both LBP and CD14 (15). Addition of FCS did not result in LPS-mediated STAT activation.

Another possible mechanism of STAT activation by LPS in the ALI model would be that STATs are activated indirectly by the cytokines generated downstream of LPS. To examine this hypothesis, the above cells were stimulated with TNF-α or IL-6, both of which are generated in mice after LPS treatment and both of which have been reported to activate the STAT transcription factors (28, 47, 82). Only IL-6 treatment resulted in STAT3 activation in both cell types (Fig. 5 and data not shown). Similar results were seen at a 24-h time point. In addition, IL-1β, alone or in combination with TNF-α, also failed to induce STAT activation in both cell types (data not shown). These results suggest that the LPS-mediated STAT3 activation in the lung is not due to the direct effect of LPS on the lung, but rather due to the indirect effect of LPS on other cell types. These results also suggest that one possible mediator of LPS-induced STAT3 activation is IL-6.
Time course of LPS-induced STAT activation in the lung vs. the liver. Others have found that STAT3 is activated in the liver secondary to systemic LPS administration (58). Determining the kinetics of liver activation relative to that observed in the lung could provide insight into the mechanism of STAT activation seen in the lung in response to LPS. STAT activation occurs in the liver within 30 min of IP LPS administration and remains elevated at 12 h (Fig. 6A). The liver kinetics indicate that STAT3 is being activated more robustly at both the earlier (30 min) and later (12 h) time points compared with that seen in the lung. To determine whether the local administration of LPS directly to the lung also resulted in STAT activation in the lung and/or the liver, experiments utilizing the IN route of LPS administration were employed. The IN administration of LPS, which has been shown by others to also induce severe ALI, similarly resulted in robust STAT activation in the lung at 2 h (Fig. 6B) (67). In addition, the local administration of LPS also resulted in intense STAT3 activation in the liver; however, in contrast to that seen with IP administration, the degree of activation in the liver was less than in the lung, as determined by densitometry. The fact that STAT activation occurs in the liver after the local administration of LPS in the lung suggests that the lung itself is orchestrating a systemic inflammatory response. Taken together, the data above suggest the possibility that the liver, the lung, and/or circulating factor(s) is contributing to the STAT activation seen in the lung in response to LPS.

STAT activation by LPS in mouse lung is redox dependent. Our laboratory has previously shown that STAT3 is a redox-regulated transcription factor both in vivo and in vitro (63, 64, and unpublished data). ALI has been shown to be accompanied by oxidative stress (22, 87). The sources of ROS in ALI have been shown to be both activated phagocytic cells as well as resident lung cells (43). To evaluate whether one mechanism of LPS-mediated STAT activation was secondary to ROS, mice were infected with adenovirus containing the cDNA for the antioxidant catalase. The IN administration of adenovirus has been previously shown by others to result in significant overexpression of exogenous proteins in the airway epithelium of mice (80). As seen in Fig. 7, catalase overexpression significantly inhibited STAT3 activation by LPS in the lung, whereas overexpression of GFP had no effect on this activation.

Fig. 5. LPS does not induce STATs in epithelial or endothelial cells in vitro. Quiescent (Q), confluent lung epithelial cells (MLE-12) were stimulated with LPS (10 μg/ml), FCS (2.5%), TNF-α (10 ng/ml), and IL-6 (50 ng/ml) for 10 min (A) or 24 h (B) as indicated. Total lysates were obtained and processed for Western blot analysis by using antibody to STAT3pTyr. The blot was stripped and reprobed with STAT3 antibody.

Fig. 6. Time course of LPS-induced STAT3 activation in the liver vs. the lung. A: BALB/c mice were treated with IP LPS for time points ranging from 30 min to 12 h before being killed. Total lysates from either the lung or liver were obtained and processed for Western blot analysis by using antibody to STAT3pTyr and STAT3. B: BALB/c mice were given 50 μg LPS intranasally and killed 2 h later. Total lysates were obtained from the liver and lung and processed by Western blot analysis by using antibody to STAT3pTyr and STAT3 as above.

Fig. 7. Overexpression of catalase prevents LPS-induced STAT activation in mouse lung. BALB/c mice were given intranasal adenovirus containing catalase (Cat; Adcat) (10⁹ particles/mouse) or green fluorescent protein (GFP; AdGFP) on days 1 and 3. On day 5, mice were given IP LPS (6 mg/kg) for 2 h before being killed. Total lysates were obtained from the lung and processed by Western blot analysis by using antibody to STAT3pTyr, STAT3, and catalase.
expression of catalase was confirmed by performing Western blot analysis on whole lung lysates. The top band represents expression of the virally encoded catalase, whereas the bottom band represents endogenous catalase. Thus LPS-mediated STAT3 activation in the lung is redox dependent.

Serum IL-6 levels have similar kinetics as STAT activation. Our cell culture experiments suggest that IL-6 may be another mechanism of STAT activation in the lung endothelium and epithelium downstream of LPS. LPS administration in mice has been demonstrated to result in increased IL-6 production in the serum as well as in multiple organs, such as the lung and liver (20, 62). In addition, IL-6, a member of the gp130 cytokine family, is a well-described STAT agonist (28). To determine whether IL-6 could be contributing to LPS-mediated STAT activation in vivo, a time course of serum IL-6 was performed by using ELISA. As shown in Fig. 8, IP LPS administration in mice resulted in a significant increase in serum IL-6 that started as early as 30 min, increased significantly at 2 and 6 h ($P < 0.05$), and started decreasing by 12 h. This pattern was also seen in the lungs but with IL-6 levels that were 10-fold lower (Fig. 8). When serum IL-6 levels are superimposed on the time course of LPS-mediated STAT3 activation, as determined by densitometry, the kinetics are nearly identical. These results suggest that IL-6 found in the serum and/or in the lungs is one possible mechanism by which LPS may induce STAT activation in the lung.

LPS-mediated STAT activation precedes lung injury and TNF-α generation. Paraffin-embedded lung sections were obtained from mice after 2, 6, 12, and 24 h of LPS treatment. Consistent with the data of others, significant lung inflammation, as manifested by neutrophil infiltration, was observed at 6 h, whereas significant lung injury, as manifested by hemorrhage and endothelial and epithelial damage, was observed at 12 h and further increased at 24 h (data not shown and Fig. 9A) (56, 73). Therefore, STAT activation is occurring before pathological evidence of ALI is present. LPS stimulation results in the generation of TNF-α, both in serum and in the lungs of mice, with subsequent neutrophil sequestration and pulmonary edema (82). Blocking TNF-α signaling has been shown by others to significantly attenuate LPS-mediated ALI (11). One possible role of STATs in mediating lung injury is the upregulation of cytokine gene expression, as others have shown that STAT3 is required for LPS-induced TNF-α expression in macrophages (13). Furthermore, in a polymicrobial sepsis model, TNF-α message was increasing before NF-κB activation was occurring, suggesting that other transcription factors were involved (8, 26). To determine whether the kinetics of STAT3 activation correlated with the increases in LPS-mediated TNF-α production, serum was obtained for ELISA. As seen in Fig. 9B, a significant increase in serum TNF-α levels was first observed at 2 h, with maximal levels seen at 6 h post-LPS treatment. Therefore, the largest increase in TNF-α generation is occurring after STAT3 activation is maximal, making this cytokine one plausible STAT target gene in mediating ALI.

**DISCUSSION**

Downstream signal transduction pathways that have been shown to participate in mediating lung inflammatory responses include the NF-κB, MAPK, and phosphotyidylinositol 3-kinase pathways (1, 33, 86). In the present study, we demonstrate that the STAT pathway is also activated in ALI induced by LPS. The STAT transcription factors are activated rapidly in the lungs (30 min) after IP LPS administration in mice with maximal activation at 1–2 h. This time course is similar to what we and others have found for LPS-mediated NF-κB activation within the lung and precedes histological signs of significant lung injury, making it an early event in the pathway from LPS to lung injury. STAT activation occurs widely throughout the lung in resident cells, as well as in the recruited inflammatory cells. We also have found that LPS activates the STAT kinases, Src and JAK, in the lung with kinetics that are consistent with STAT activation. Importantly, we determined that STAT activation occurred in a gastric acid aspiration model, as well as an
acute pancreatitis model of ALI. This suggests that STATs may be a common pathway for mediating ALI, regardless of the inciting factor. This concept is further supported by the work of others showing that STAT activation occurs in lungs of a resuscitative hemorrhagic shock model (30). Finally, STAT activation in the lung occurs long before signs of ALI, suggesting that the STAT transcription factors may play an important role in initiating the inflammatory response in the lungs.

The mechanism of STAT activation by LPS in the lung likely involves a complex interplay between inflammatory cells, such as macrophages, and resident lung cells. Phagocytic cells have been implicated in the upregulation of transcription factors and cytokines in ALI, and macrophages are likely the first cells to respond directly to LPS (21, 88). In addition, there is evidence that the lung cells (epithelium and endothelium) as well as various organs, such as the liver, are capable of directly responding to inflammatory stimuli, such as endotoxin, with transcription factor activation and the subsequent elaboration of cytokines and inflammatory mediators (3, 5). After LPS administration, STAT activation was first observed in the central and subsequently the distal airway epithelium before appearing in the rest of the lung, suggesting that the airway is actually playing a key role in initiating the inflammatory response in the lungs. Consistent with this idea are data from others demonstrating that the airway epithelium itself can orchestrate immune and inflammatory responses via the upregulation of integrin receptors, cytokines, chemokines, and oxidants (70, 72). In addition, maximal STAT3 activation in the lung occurs after that seen in the liver, raising the possibility that the liver acute-phase response may be contributing to STAT activation in the lung or possibly amplifying it. The fact that STATs are also found to be activated in the liver after local administration of LPS in the lung suggests the existence of common circulating mediators that lead to STAT activation.

It is likely that LPS does not directly activate STATs in lung cells, as our cell culture experiments failed to show STAT activation by LPS. A plausible mechanism of STAT activation by LPS is that it occurs indirectly via cytokines or possibly ROS that are generated downstream of LPS. Our laboratory has previously demonstrated that STATs are activated by ROS, and others have determined that ALI is characterized by oxidative stress (59, 63, 64). We now have demonstrated that LPS-mediated STAT3 activation similarly requires ROS in mouse lung.

Our work also suggests that IL-6 is a possible candidate for activation of STATs, because it directly activated STAT3 in the epithelial and endothelial cells, whereas TNF-α, IL-1β, or a combination of both cytokines failed in this regard. Possible sources of IL-6 production include macrophages, the liver, and the lung itself (16, 23). In addition, the kinetics of IL-6 generation after LPS treatment suggest that IL-6 may be one mediator of STAT activation by LPS in the lungs. Work by others, however, suggests that LPS-mediated STAT3 activation may be IL-6 independent because it still occurred in the liver of IL-6−/− mice treated with LPS (3). Whether LPS activation of STATs in the lung is IL-6 dependent is unknown and will be the basis of future work using IL-6-deficient mice.

We also determined that LPS induces the STAT kinases Src and JAK2 in the lung with kinetics, consistent with STAT activation. Our and other’s data show that Src and JAK, in some cases, reside in a common pathway, with Src upstream of JAK activation (12, 64, 76). Future studies will need to determine whether JAK and/or Src kinases are in fact required for STAT activation in the lung in response to LPS and whether this regulation of STATs varies, depending on the inciting ALI.

**Fig. 9.** STAT3 activation precedes LPS-induced lung injury and TNF-α generation. A: representative histological section of the lungs of mice treated with IP PBS (control) or LPS for 24 h by using hematoxylin and eosin staining. B: BALB/c mice were treated with IP LPS (6 mg/kg) for the indicated time points before being killed. Serum was obtained via right heart puncture and processed for ELISA by using TNF-α antibody. Values are means ± SE of 4 animals. *P < 0.05 compared with PBS.
stimulus. Determining the kinases required for STAT activation in this model and other models of ALI is critical for future studies aimed at identifying the role of the STAT pathways in ALI and may provide the basis of novel therapeutic targets for ALI treatment. Data from others have already demonstrated that mice deficient in Src kinase or JAK kinase family members are resistant to endotoxin-mediated shock (36, 45). Furthermore, others have shown that LPS-induced ALI and mortality could be attenuated by pretreating animals with tyrosine kinase inhibitors, although the precise kinase through which these inhibitors have their effects is not entirely clear (49, 57). These studies also suggest that the inhibitors may be exerting their effects through blockade of the NF-κB and/or MAPK pathways (34, 44). These tyrosine kinase inhibitors may also be exerting a beneficial effect by preventing the activation of the STAT transcription factors via the inhibition of the key STAT kinases Src and JAK.

Our data indicate that STAT activation occurs long before significant lung injury is present. This rapid activation, 30–60 min post-LPS administration, also occurs before significant generation of the proximal proinflammatory cytokine TNF-α, suggesting that STAT3 may be playing a critical role in initiating pulmonary inflammation in ALI. STAT3 has already been shown to be essential for the liver’s response to LPS and IL-6 via the activation of downstream acute-phase genes, such as TNF-α, IL-1, and haptoglobin (4). Candidate STAT target genes that could contribute to the pathogenesis of ALI include cytokines, chemokines, adhesion molecules, and inflammatory mediators, such as iNOS or cyclooxygenase. The fact that STATs also are involved in the upregulation of LPS signaling molecules, such as LBP and MD-2, suggests that STATs may also lead to amplification of inflammation in sepsis (2, 38). STATs, depending on the cellular context, have been shown to be vital for modulating both pro- and anti-inflammatory responses. In addition, the STAT pathway has been shown to both enhance and downregulate NF-κB-mediated gene expression (51, 52, 77).

That the role of STATs in ALI is likely to be complex is further confirmed by studies of genetically manipulated mice. Determining the role of STAT3 in mediating inflammatory responses has been hampered by the fact that knocking out STAT3 results in embryonic lethality (69). Cell-specific disruption of STAT3 in mice in myeloid or endothelial cells, however, led to enhanced susceptibility to endotoxin-mediated inflammation (35, 68). Conversely, SOCS-1-deficient mice showed an increased sensitivity to LPS, suggesting that unchecked STAT activation in multiple cell types results in an overall proinflammatory state (48). Finally, mice generated with a knockout of STAT3β, a splice variant of STAT3 that can exert positive or negative effects on gene expression, had impaired recovery from LPS-induced shock, indicating that STAT3β is likely important for downregulating LPS-mediated responses (85). Future work will need to be done to confirm the role of the LPS-induced STAT3 found in the lung in mediating ALI. We expect from our present studies that more global inhibition of the STAT pathway, in multiple cell types, will have a beneficial effect, because this pathway appears to be an early event in the host inflammatory response to LPS as well as other inciting stimuli.

While the clinical and pathological entity of ALI is the same, regardless of the inciting stimulus, it is likely that the signaling pathways that initiate the process are quite distinct. These signaling differences may be one explanation why no immune-modulating therapies have been effective in clinical trials for ALI, which have grouped all patients together, regardless of the etiology of their disease. Our work suggests that understanding the STAT pathway is critical for understanding the mechanisms of ALI because it is critical for the function of cytokines and immune regulation, as well as closely interacting with other signaling pathways. It is likely that a better understanding of the complex signaling pathways that are activated in ALI may promote the development of novel and efficacious therapies.

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REFERENCES

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