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*J Immunol* 2012; 188:4535-4542; Prepublished online 26 March 2012;
doi: 10.4049/jimmunol.1003655
http://www.jimmunol.org/content/188/9/4535
Inhibition of Mammalian Target of Rapamycin Augments Lipopolysaccharide-Induced Lung Injury and Apoptosis

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Acute lung injury during bacterial infection is associated with neutrophilic inflammation, epithelial cell apoptosis, and disruption of the alveolar-capillary barrier. TLR4 is required for lung injury in animals exposed to bacterial LPS and initiates proinflammatory responses in part via the transcription factor NF-κB. Ligation of TLR4 also initiates a proapoptotic response by activating IFN-β and STAT1-dependent genes. We recently demonstrated that mammalian target of rapamycin (mTOR), a key controller of cell growth and survival, can physically interact with STAT1 and suppress the induction of STAT1-dependent apoptosis genes. We therefore hypothesized that the mTOR inhibitor rapamycin would increase LPS-induced apoptosis and lung injury in vivo. Rapamycin increased lung injury and cellular apoptosis in C57BL/6j mice exposed to intratracheal LPS for 24 h. Rapamycin also augmented STAT1 activation, and the induction of STAT1-dependent genes that mediate cellular apoptosis (i.e., Fas, caspase-3). LPS-induced lung injury was attenuated in STAT1 knockout mice. In addition, LPS and IFN-β–induced apoptosis was absent in cultured cells lacking STAT1, and, unlike in wild-type cells, a permissive effect of rapamycin was not observed. In contrast to its effect on STAT1, rapamycin inhibited NF-κB activation in vivo and reduced selected markers of inflammation (i.e., neutrophils in the bronchoalveolar lavage fluid, TNF-α). Therefore, although it inhibits NF-κB and neutrophilic inflammation, rapamycin augments LPS-induced lung injury and apoptosis in a mechanism that involves STAT1 and the induction of STAT1-dependent apoptosis genes. The Journal of Immunology, 2012, 188: 4535–4542.

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cute lung injury in patients with viral or bacterial pneumonia accounts for significant morbidity and mortality in hospitalized patients. The pathogenesis of acute lung injury is complex and involves the recruitment of neutrophils, the elaboration of inflammatory cytokines, and apoptosis of epithelial cells. Together, these lead to disruption of the alveolar epithelial barrier, pulmonary edema, and abnormalities in gas exchange (1).

In animal models of Gram-negative sepsis or pneumonia, the initiation of lung injury requires ligation of the TLR4 by bacterial LPS and the induction of proinflammatory and proapoptotic transcriptional programs (2). Known signal transduction pathways downstream of TLR4 include myeloid differentiation primary response gene 88 (MyD88)–dependent and independent signaling intermediates (3). Via MyD88, TLR4 activates the transcription factor NF-κB and the transcription of proinflammatory and survival genes. However, TLR4 also triggers the MyD88–independent activation of IFN regulatory factor 3 (IRF-3), a transcription factor that rapidly induces the production of IFN-β (IFN-β). IFN-β then induces apoptosis and antimicrobial genes in a feed-forward autocrine-paracrine signaling pathway that requires the transcription factor STAT1. STAT1 is required for the synthesis of several key apoptosis regulators that mediate lung injury, including CD95/Fas and inducible NO synthase (1, 4, 5). Genetic engineered mice with defects in IFN-β (6) or STAT1 (7) exhibit deficient antibacterial innate immune responses.

Consistent with an important role for apoptosis in acute lung injury, signaling pathways that regulate cell survival and proliferation have been implicated in the genesis of acute lung injury (e.g., PI3K, NF-κB, p53) (8–10). One such signaling intermediate is mammalian target of rapamycin (mTOR), a highly conserved and ubiquitously expressed controller of cell growth, proliferation, and survival (11). mTOR senses growth or metabolic signals (e.g., ATP, oxygen, amino acids, glucose, reactive oxidant species) and exerts anabolic effects by stimulating protein synthesis and ribosomal biogenesis, enhancing cell proliferation, and promoting cell survival (12). Rapamycin (i.e., sirolimus [Rapamune]) is a specific inhibitor of mTOR and is a commonly used pharmacologic tool for the study of mTOR biology. In addition, rapamycin is approved by the U.S. Food and Drug Administration for immunosuppression in transplant patients, cancer chemotherapy, and local prevention of coronary artery stent thrombosis (13). In this study, we used rapamycin to dissect the relative contribution of mTOR during the evolution of LPS-induced lung injury.

In regard to protein synthesis and cell growth, known mTOR effectors include p70 S6 kinase (S6K), 4E-BP1, and Akt; however, mTOR can also modify gene transcription by directly regulating transcription factors (11). For example, by stimulating NF-κB, mTOR potentiates cell survival responses (14). We recently iden-
ified a physical and functional interaction between mTOR and STAT1 (15). In intact cells, inhibition of mTOR kinase activity lead to increased STAT1 nuclear content and transcription of IFN-sensitive apoptosis genes (16). Based on these studies, we hypothesized that mTOR blockade with rapamycin would augment LPS-induced injury and apoptosis in the lung, as well as the STAT1 transcriptional response in vivo. In this study, we show that rapamycin increases acute lung injury and apoptosis in mice exposed to LPS. Increased injury and cell death were associated with elevated levels of activated STAT1 and STAT1-independent apoptosis genes, but reduced NF-kB activation and markers of inflammation (e.g., neutrophils and TNF-α in the bronchoalveolar lavage fluid). In vivo and in cultured cells, STAT1 was required for LPS-induced lung injury and apoptosis, and the proapoptotic effect of rapamycin was absent in STAT1-deficient cells. Our findings indicate that mTOR is an endogenous suppressor of STAT1, proapoptotic responses, and lung injury, independent of NF-kB-mediated proinflammatory responses.

Materials and Methods

Materials

STAT1-deficient (U3A) cells and their wild-type control (2fTGH) were obtained from Dr. G. Stark (Cleveland Clinic) and propagated as described previously (17). Escherichia coli O55:B55 LPS was purchased from Sigma-Aldrich. IFN-γ was obtained from PBL IFNSource. Rapamycin was purchased from Biomol. Hexadecyltrimethylammonium bromide and o-dianisidine dihydrochloride were purchased from Sigma-Aldrich. Abs against phospho-p70 S6 kinase (pS6K Thr 389), p70 S6 kinase (S6K), phospho-S6 (Ser 235/236), phospho-p65 (Ser 536), IκBα, S6, and cleaved caspase-3 were purchased from Cell Signaling Technology. The Ab against phospho-STAT1 (pSTAT1 Ser 727) and caspase-3 were purchased from Millipore and Becton Dickenson, respectively. Anti–β-tubulin, β-actin, and anti STAT1 Abs were purchased from R&D Systems.

Animal preparation

Animal protocols were approved by the animal care committee at McGill University (Montreal, Canada). Six-week-old male C57BL/6J, BALB/c STAT1+/- or BALB/c STAT1-/- mice were injected i.p. with rapamycin (1.5 mg/kg) or control 6 h before tracheotomy and intratracheal instillation of saline or LPS (50 μl of 1 mg/ml solution). There were no perioperative deaths, and animals were killed by CO2 asphyxiation 24 h later for assays of lung injury, apoptosis, and gene expression, as indicated below. BALB/c and C57BL/6J wild-type mice were obtained from Harlan and Charles River, respectively. STAT1-deficient (U3A) cells and their wild-type control (2fTGH) were obtained from Dr. J. Durbin (18) and obtained from Dr. A. Koromilas (McGill University). Genotyping of STAT1 alleles was performed on mouse ear punch samples with the following primers:

- Forward primer 5’-CTATGGTTTCTATGTTGATATC-3’, (reverse) 5’-GAGATAATTCACAAAAATCGAGAG-3’;
- Forward primer 5’-CTGATCCAGGCAGCGTTG-3’, (reverse) 5’-ACCCTCCAGGCAGCGTTT-3’.

These primers were used in a single PCR reaction to identify the wild-type allele (product size 142 bp) or the knockout allele (product size 342 bp).

Measures of lung injury

For lung wet-to-dry ratio, the lungs were removed en bloc, gently blotted to remove excess blood, weighed, and dried at 60˚C for 72 h. The wet-to-dry mass ratio was calculated for each excised lung. For histopathology analysis. For measures of alveolar-capillary leak, the Evans Blue (EB) dye technique was used as described previously (19). Tetrasodium salt of EB dye was diluted to a concentration of 5 mg/ml in Dulbecco’s PBS (pH 7.4). Whole lung lysates were measured by spectrophotometry. Dilution standards for EB and blank samples were prepared in 50% formamide, and a lung-specific correction factor was applied to the homogenate values (A100nm/EB = A100nm/A620 + 0.004) as described (19).

Measures of apoptosis

For TdT-mediated dUTP nick end labeling (TUNEL) assay, histopathology slides were rehydrated in PBS for 10 min before boiling in citrate buffer (100 mM citrate, pH 6.0, 0.05% Tween) for 5 min. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer’s protocol. Coverslips were applied using mounting solution (Dako Cytomation) containing the nuclear stain DAPI, and fluorescence was detected using an Olympus ×70 inverted fluorescence microscope. The number of TUNEL (FITC)-positive cells per high-power field (magnification ×400) was determined after acquisition of images using charge-coupled device camera and Metamorph software. For detection of cleaved caspase-3, immunohistochemical detection was performed on a Discovery XT automatic immunohistochemistry (Ventana Medical Systems). Following deparaffinization and Ag retrieval, slides were incubated for 2 h at room temperature with 1:100 anti-cleaved caspase-3 Ab (Biocare Medical) in PBS, washed and incubated with biotin-conjugated anti-rabbit Ab (Jackson ImmunoResearch). Streptavidin-HRP, 3,3-diaminobenzidine detection kit, and avidin-biotin blocking kit were used according to the manufacturer’s instructions (Ventana Medical Systems). The sections were counterstained with hematoxylin before application of a bluing reagent.

Assessment of bronchoalveolar lavage and lung neutrophil content

Bronchoalveolar lavage (BAL) fluid was obtained by cannulating the trachea and then lavaging the lungs four times with 0.5 ml ice-cold PBS. BAL fluid samples were pooled for each mouse and centrifuged onto glass slides at 1000 rpm for 10 min using Shandon Cytospin 2. The slides were fixed and stained with Diff Quick (Dade Behring) before counting the number of neutrophils per high-power field (400 × magnification) by light microscopy.

For lung neutrophil content, myeloperoxidase activity was measured in whole lung lysates as described previously (20). Lung samples were homogenized in homogenization buffer (0.5% hexadecytrimethylammonium bromide, 5 mM EDTA, 50 mM potassium phosphate [pH 6.0], 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) before sonication (three 20-s pulses), and centrifugation at 3000 × g for 30 min. After measurement of protein concentration (Bradford assay), 10 μl of each sample was added to 250 μl assay buffer (0.005% hydrogen peroxide, 0.5 mM o-dianisidine dihydrochloride, 100 mM potassium phosphate [pH 6.0]), before measurement of absorbance at 460 nm every 20 s for 10 min by spectrophotometry. Data for myeloperoxidase activity are presented as fold change in relative absorbance units per second per microgram of protein.

Detection of lung mRNA

Whole lungs of lavaged mice were removed en bloc and then stored at −20°C in RNA Later (Invitrogen) prior to RNA extraction. Lungs were mechanically disrupted in 1 ml Trizol reagent (Invitrogen) per 50–100 μg of tissue using a brickman homogenizer. RNA was purified by phenol-chloroform extraction per the manufacturer’s protocol. cDNA was generated by reverse transcription from 2 μg RNA (Superscript II; Invitrogen). Real-time PCR was performed on 1 μl cDNA using Power SYBR Green Universal PCR master mix (AB), per the manufacturer’s instructions. The following sequences were used for forward and reverse real-time PCR primers: Fas (forward) 5’-AGGACCTGCAAAATGATAGGG-3’, (reverse) 5’-GGTGTC-AATGTTGTTCCACT-3’; caspase-3 (forward) 5’-CATTTATGGGACAATGGGC-3’, (reverse) 5’-CCGGTCTTGTTAGTTTCTCA-3’; 45S rRNA (forward) 5’-GACCGCTGTCCTCTTCACT-3’, (reverse) 5’-GTTGATACCCAGAATAGC-3’. PCR reactions were carried out for 45 cycles (ABI 7500 Real Time PCR System). Results are expressed as fold induction in mRNA levels (SEM) as calculated by the ΔΔCt method (21).
Detection and quantification of protein levels

Whole lungs of lavaged mice were removed en bloc, snap frozen in liquid nitrogen, and stored at −80°C prior to protein extraction. Lungs were mechanically disrupted using a Brickman mechanical homogenizer in homogenization buffer (20 mM Tris [pH 8.0], 0.5% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 50 mM NaF; 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μM sodium orthovanadate). Homogenates were snap frozen on dry ice, thawed, and cleared by centrifugation at 16,000 × g for 30 min at 4°C. For detection of proteins in cultured cell lines, 2fTGH or U3A cells were washed once with cold PBS and incubated for 15 min on ice in homogenization buffer. After freezing and thawing, cells were homogenized on ice and cleared (1000 × g for 5 min). Supernatants were further separated (16,000 × g for 30 min) to generate particulate-free lysates. For mouse lung homogenates and cultured cell lysates, supernatants were assayed for protein content by Bradford assay. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane before immunoblotting with primary Abs as indicated. Membranes were incubated with anti-rabbit or anti-mouse IgG HRP conjugated Abs and developed using Super-Signal West Pico chemiluminescence detection kit (Pierce). Images of Western blot films were acquired using an Alpha Imager (Innotech), and analyzed with Alpha Ease FC software (version 4.1.0). Integrated band density for the indicated protein was obtained using the spot density and auto-background functions, and was normalized to that of β-tubulin or β-actin for each sample. Levels of TNF-α in the BAL fluid were determined using a commercially available ELISA kit (R&D Systems), according to the manufacturer’s instructions.

Detection of cell number in vitro

U3A or 2fTGH cells were washed twice with PBS before incubating with 0.2% crystal violet in 25% methanol for 15 min. Excess stain was removed by washing four times with PBS. Stained cells were solubilized by mixing with 1% SDS solution for 15 min. The OD of each sample (100 μl) was determined at 570 nm and 620 nm using a SpectraMax M2 microplate reader (Molecular Devices).

Statistical analysis

Statistical analysis was performed using JMP 8.0.1 software (SAS Institute) Student t test, or ANOVA with Tukey–Kramer multiple comparisons tests was applied to grouped data for each experiment. A p value < 0.05 was considered statistically significant.

Results

Rapamycin enhances LPS-induced lung injury

C57BL/6J mice were exposed to i.p. rapamycin (1.5 mg/kg), 6 h before intratracheal administration of saline or E. coli LPS (50 μg) for 24 h. There was no mortality observed in any of the treatment groups. In agreement with previous studies, LPS-treated mice exhibited histopathologic evidence of lung injury characterized by infiltration of inflammatory cells, hemorrhage, thickened alveolar septae, and proteinaceous exudates (Fig. 1A). Consistent with

FIGURE 1. Rapamycin enhances LPS-induced lung injury. Six-week-old male C57BL/6J mice were pretreated without or with rapamycin (1.5 mg/kg i.p.). After 6 h, saline or 50 μL LPS was administered intratracheally. After an additional 18 h, mice were asphyxiated with CO2 gas. (A) Formalin-fixed lung sections were stained with H&E. Original magnification ×400. Intra-alveolar hemorrhage (black arrow) and proteinaceous exudate with cellular infiltrate (red arrow) are indicated. (B) Lung injury was measured by lung wet to dry tissue mass (mean wet to dry ratio ± SEM; n = 7 mice per group). (C) Total cell and neutrophil numbers in BAL fluid were detected by Diff-Quick staining after preparation of cytopsin slides (mean neutrophil number ± SEM; n = 5 mice per group). (D) Whole lung myeloperoxidase activity was assessed as outlined in Materials and Methods. Data are means of fold change versus vehicle-treated animals = 1 (means ± SEM; n = 3 mice per group). Data were analyzed by one-way ANOVA (p < 0.05; n = 5 mice), followed by Tukey–Kramer multiple post hoc comparisons test, and are representative of two individual experiments. *p < 0.05 versus control, †p < 0.05 versus LPS alone.
intratracheal administration, the injury was not observed homogeneously throughout the lungs. In contrast to a previous report (22), there was no inhibitory effect of rapamycin in LPS-induced lung injury by histopathology.

To quantify lung injury, we next determined the effect of rapamycin on LPS-induced pulmonary edema by whole-lung wet-to-dry ratio. Consistent with previous studies, wet-to-dry ratio was 3.7 in control animals and increased by 17% in LPS-exposed animals (Fig. 1B). Administration of rapamycin led to a statistically significant increase in lung wet-to-dry ratio in animals exposed to LPS (4.5 versus 4.2; \( p < 0.05 \)). In contrast to lung injury, the number of neutrophils in the BAL fluid and lung myeloperoxidase activity was reduced by rapamycin, suggesting the involvement of a mechanism other than neutrophilic recruitment or inflammation (Fig. 1C, 1D).

**Rapamycin enhances LPS-induced cellular apoptosis**

Previous studies demonstrated a requirement for apoptosis and the death receptor Fas/CD95 in LPS-induced lung injury (23, 24). Moreover, we recently reported the suppression of STAT1-dependent genes (e.g., Fas, caspase-1, inducible NO synthase) by mTOR (16), and reasoned that inactivation of mTOR would enhance apoptosis in vivo. In the current study, administration of intratracheal LPS significantly increased levels of cleaved caspase-3 (8.4 versus 4.2 positive cells per high-power field; \( p < 0.05 \); Fig. 2A). Rapamycin further enhanced cleaved caspase-3 levels in animals exposed to LPS (13.6 versus 8.4 positive cells per high-power field; \( p < 0.05 \)). By TUNEL staining, LPS increased cellular apoptosis compared with that in control animals (2.5 versus 0.3 TUNEL-positive cells per high-power field; \( p < 0.05 \); Fig. 2B). Rapamycin further increased apoptosis in LPS-treated mice (4.4 versus 2.5 TUNEL-positive cells per high-power field; \( p < 0.05 \)). These results indicate that the augmenting effect of rapamycin on LPS-induced lung injury correlates with its effect on apoptosis in the lung.

**STAT1 is required for the enhancing effect of rapamycin on cellular apoptosis**

We previously demonstrated an enhancing effect of rapamycin on the induction of STAT1-dependent proapoptotic genes (16). To demonstrate that STAT1 is required for the effect of rapamycin on LPS and IFN-\( \beta \)-induced cellular apoptosis, we used a STAT1-deficient human cell line (U3A) and its wild-type control (2fTGH). Inhibition of mTOR activity with rapamycin, or incubation with LPS and IFN-\( \beta \), significantly increased cleaved caspase-3 levels in wild-type (2fTGH) cells (Fig. 3A, lanes 4 and 5). The addition of LPS and IFN-\( \beta \) further increased cleaved caspase-3 levels in rapamycin-treated cells compared with cells exposed to rapamycin alone (Fig. 3A, lane 6 versus 4). In contrast, rapamycin failed to significantly induce or enhance cleavage of caspase-3 in STAT1-deficient (U3A) cells (Fig. 3A, lanes 10 and 12). Corresponding densitometry data demonstrate statistically significant augmentation of LPS/IFN-\( \beta \)-induced apoptosis in control, but not STAT1-deficient, cells (Fig. 3A, bottom panel).

We next used crystal violet staining to quantify changes in cell number in cultures exposed to rapamycin, LPS/IFN-\( \beta \), or both. In wild-type cells exposed to rapamycin for 40 h, there was a marked decrease in cell number, likely because of its known inhibitory effect on cell proliferation (Fig. 3B) (25). The addition of LPS/IFN-\( \beta \) to rapamycin led to further decreased cell number, which correlated with increased cleaved caspase-3 levels (Fig. 3A, lane 6). LPS and IFN-\( \beta \) did not decrease cell number in STAT1-deficient cells, perhaps because of a failure to induce apoptosis (Fig. 3A, lane 11). In contrast to control cells, LPS and IFN-\( \beta \) did not further decrease cell number when added to rapamycin-treated STAT1-deficient cells (Fig. 3B). These results indicate that the incremental effect of LPS and IFN-\( \beta \) on rapamycin-induced apoptosis, and reduction in cell number, requires STAT1.

To demonstrate that STAT1 is required for LPS-induced lung injury in vivo, we used BALB/c STAT1 knockout (STAT1 \(-/-\)) or wild-type (STAT1 \(+/+\)) mice exposed to intratracheal LPS. Moreover, we measured intravascular EB dye transit from the vasculature into the alveolar space (i.e., alveolar capillary leak) as another index of acute lung injury. In wild-type mice, intratracheal LPS led to a significant increase in EB dye leak (Fig. 3C); the LPS-induced leak was absent in STAT1 \(-/-\) mice.

**Rapamycin enhances the expression of genes involved in apoptosis and lung injury**

Through TLR4, LPS leads to the activation of STAT1-dependent apoptosis genes (26, 27). We therefore determined the effect of rapamycin on the expression of Fas and caspase-3 mRNA. Consistent with previous studies, expression of Fas was increased, and that of caspase-3 reduced, 24 h after exposure of mice to LPS (Fig. 4) (28, 29). In mice exposed to LPS, rapamycin significantly increased Fas mRNA levels compared with mice exposed to LPS alone. Similarly, rapamycin prevented the reduction in caspase-3 mRNA levels observed in LPS-treated mice.
Rapamycin enhances the activation of STAT1

The proapoptotic effects of TLR4 receptor ligation are mediated primarily by the induction of IFN-β and autocrine-paracrine activation of STAT1 (26, 30–32). We reasoned that the increase in STAT1-dependent genes by rapamycin in vivo (Fig. 4) correlates with additional activation of STAT1. By Western blot analysis of whole lung homogenates, the levels of phosphorylated (activated) and total STAT1 were significantly increased in mice exposed to LPS for 24 h (Fig. 5A, lane 2 versus 1). Phospho-STAT1 and total STAT1 levels were further increased by rapamycin (lane 4 versus 2). As was the case for caspase-3 (Fig. 4), STAT1 mRNA levels were increased in mice exposed to LPS and rapamycin, but not LPS alone (Fig. 5B). Thus, the additional enhancement of lung injury, apoptosis, and induction of apoptosis genes caused by rapamycin correlates with elevated levels of activated STAT1 in mice exposed to LPS.

Rapamycin attenuates the activation of NF-κB by LPS

In contrast to STAT1, previous work demonstrated inhibition of NF-κB by rapamycin, NF-κB–dependent inflammatory mediators, and neutrophil recruitment (14, 22). We therefore confirmed that rapamycin blocks NF-κB activation in mice exposed to LPS and rapamycin, despite the observed increase in STAT1 activation and apoptosis. As expected, LPS increased phosphorylation of the NF-κB p65 subunit, and reduced IkBα levels; rapamycin blocked LPS-induced phosphorylation of p65 and reversed the degradation of IkBα (Fig. 6A, 6B). In agreement with previous studies, LPS increased BAL TNF-α protein levels (33). Consistent with its effect on neutrophil recruitment and NF-κB signaling, rapamycin inhibited TNF-α induction by LPS when measured in the BAL fluid (Fig. 6C). These results indicate that rapamycin promotes a proapoptotic, STAT1-dependent transcriptional program while suppressing inflammatory modulators such as NF-κB, neutrophils, and TNF-α.

Rapamycin attenuates mTORC1 effectors and ribosomal biogenesis

mTOR nucleates two protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (12). Rapamycin potently and specifically inhibits mTORC1, which controls cell growth and ribosomal biogenesis by activating p70 S6 kinase. The rapamycin-insensitive mTORC2 controls cell proliferation and cytokinesis in part by activating Akt and protein kinase C-α (34, 35). We next determined whether the effect of rapamycin on lung injury and apoptosis accompanied blockade of mTORC1. In con-

**FIGURE 3.** STAT1 is required for rapamycin enhancement of LPS and IFN-β–induced apoptosis in vitro and LPS-induced lung vascular leak in vivo. 2TGH or U3A cells were incubated with LPS or IFN-β, or both, in the absence or presence of rapamycin for (A) 24 h or (B) 40 h. In (A), cleaved caspase-3 levels (CCasp3) in cell lysates were assessed by Western blot. Data were quantified by band densitometry (means of cleaved caspase-3 integrated pixel density normalized to β-actin ± SEM; n = 3 experiments). Means were significantly different by two-way ANOVA (p < 0.0001). *p < 0.05 versus LPS or control, †p < 0.05 versus LPS/IFN-β by Tukey–Kramer multiple post hoc comparisons test. In (B), to assess cell number, cells were stained with crystal violet and the OD of solubilized dye was measured. Changes in OD at 570 nm after correction for the OD at 620 nm are shown (mean ± SEM; n = 4 independent experiments each performed in triplicate). Means were significantly different by two-way ANOVA (p < 0.01). By Tukey–Kramer multiple post hoc comparisons test: *p < 0.05 versus control; †p < 0.05 versus rapamycin alone or control; ns, not significant versus rapamycin alone. (C) Wild-type (STAT1+/+) or knockout (STAT1−/−) mice were exposed to saline or 50 μg LPS administered intratracheally. After 22 h, EB dye was injected systemically, before asphyxiation with CO₂ gas 2 h later. EB dye leak was measured as described in Materials and Methods. Data are expressed as micrograms of EB dye per gram of lung (mean ± SE; n = 8–10 mice per group). *p < 0.05 versus control by Student t test.
the activation of the proinflammatory and antiapoptotic transcription factor NF-κB was attenuated. In cultured cells and mice, STAT1 was required for apoptosis and lung injury, respectively. Rapamycin did not cause or permit apoptosis in STAT1-deficient cells. These results indicate that inactivation of mTOR can sensitize the proapoptotic response to bacterial products and that enhanced lung injury is one manifestation. Consistent with this observation, complications of rapamycin therapy include conditions characterized by dysregulated lung injury and repair such as interstitial pneumonitis, bronchiolitis obliterans organizing pneumonia, and pulmonary fibrosis (36). Moreover, other conditions that reduce mTOR activity, such as nutritional deprivation, might also predispose to tissue injury during microbial infection.

Using rapamycin to dissect the role of mTOR in acute lung injury, our studies reveal an important role for STAT1 in the in vivo and in vitro proapoptotic effects of LPS. Previous studies showed that physical interactions between TLR4 and its signaling adaptor proteins form the molecular basis for these distinct responses (3). Ligation of TLR4 by LPS recruits MyD88, which in turn activates NF-κB and proinflammatory genes. In addition to MyD88, recruitment of the adaptor protein TIR-domain–containing adapter–inducing IFN-β activates IRF-3 and the synthesis of IFN-β. The subsequent activation of STAT1 is required for the induction of MyD88–independent genes induced by LPS (e.g., IP10, RANTES, IRF-1) (37). Our data indicate that inactivation of mTOR enhances MyD88–independent signaling (i.e., STAT1, apoptosis) while attenuating MyD88–dependent processes (i.e., NF-κB, neutrophil recruitment, TNF-α release).

Inhibition of mTOR promoted STAT1 activation under conditions of attenuated S6K activity (Fig. 7), suggesting a direct effect of mTOR on transcriptional control mechanisms. The effect of mTOR on IFN signaling and innate immune function was previously thought to occur primarily via its effectors p70 S6 kinase or 4E-BP1 and the control of protein synthesis (38, 39). However, recent studies indicated a direct molecular link between mTOR and transcription factors. Inactivation of mTOR enhanced STAT1 nuclear levels in human lung epithelial cells and amplified the induction of STAT1–dependent apoptosis genes by IFN-γ (16). mTOR also regulated NF-κB, although its effect depended on the cell type or model. In vitro studies using cultured monocytes exposed to rapamycin revealed increased NF-κB activity and TNF-α levels; in contrast, NF-κB and TNF-α were suppressed by rapamycin in the lungs of mice exposed to LPS (22, 40). In a separate study, rapamycin increased mortality in mice exposed to i.p. LPS, and this was associated with enhanced levels of caspase-1, an NF-κB–dependent protein, but decreased TNF-α levels (41). In this study, we investigated STAT1 and NF-κB simultaneously, and
confirmed inhibition of NF-κB by rapamycin in vivo. However, in contrast to NF-κB, rapamycin augmented STAT1 activity and the transcription of STAT1-dependent apoptosis genes.

The augmenting effect of rapamycin on lung injury and apoptosis correlated with activation of STAT1, and inhibition of NF-κB. Of note, a recent report also described an inhibitory effect of rapamycin on NF-κB, TNF-α, and neutrophil recruitment in LPS-treated mice (22); however, despite the expected antisurvival effect of NF-κB blockade, and in contrast to the current study, lung injury was reported to be blocked by rapamycin. Possible explanations for this discrepancy include differences in the timing of rapamycin administration and high dose (two 5-mg/kg doses 6 h prior to and concurrent with LPS administration in the previous study versus one 1.5-mg/kg dose 6 h prior to LPS in the current study). Moreover, the former report focused on neutrophilic inflammation and did not address MyD88-independent TLR4 signaling responses or cellular apoptosis. Finally, our in vivo and in vitro data demonstrate that STAT1 is required for LPS-induced lung injury and apoptosis (Fig. 3). The data also support an inhibitory role for mTOR in the induction of STAT1- and LPS-induced tissue injury. Further genetic dissection of the effects rapamycin in vivo will be required to reconcile the disparate findings in regard to lung injury.

Although both neutrophils (neutrophil hypothesis) and epithelial cell death (epithelial cell hypothesis) appear to play a role, there is a debate on the relative importance of each (42). In the current study, the inhibitory effect of rapamycin on NF-κB, TNF-α, and bronchoalveolar fluid neutrophil levels did not correlate with its enhancement of apoptosis, STAT1, or lung injury. Moreover, the inhibitory effect of rapamycin on NF-κB and TNF-α might be consistent with enhanced lung injury given the known pro-survival (i.e., antiapoptotic) effects of NF-κB and TNF-α (43).

In addition to effects on cell survival, the TNF-α lectin-binding domain reduced acute lung injury in part by promoting alveolar liquid clearance (33). Our results suggest that mTOR selectively suppresses MyD88-independent signaling and cellular apoptosis. This paradigm is supported by in vitro studies demonstrating a physical and functional association between mTOR and STAT1 (16). Other inhibitors of mTOR activity include reduced essential amino acids, glucose, oxygen, or growth factors. The mTOR-STAT1 signaling axis may therefore represent an important in-
terface between metabolism, innate immunity, and lung injury in vivo.

Acknowledgments
We thank Dr. S. Levine and Dr. J. Moss (National Institutes of Health) for advice and critical review of the manuscript, Dr. A. Koromilas for providing STAT1 knockout mice, I. Angers for conducting Evans Blue dye leak experiments, and Dr. S. Vidal (McGill University) for providing real-time PCR primer sequences.

Disclosures
The authors have no financial conflicts of interest.

References