Stimulation of Signal Transducer and Activator of Transcription-1 (STAT1)-dependent Gene Transcription by Lipopolysaccharide and Interferon-γ Is Regulated by Mammalian Target of Rapamycin*

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Mammalian target of rapamycin (mTOR) and phosphatidylinositol 3-kinase (PI3K) regulate cell growth, protein synthesis, and apoptosis in response to nutrients and mitogens. As an important source of nitric oxide during inflammation, human inducible nitric oxide synthase also plays a role in the regulation of cytokine-driven cell proliferation and apoptosis. The role of mTOR and PI3K in the activation of human inducible nitric oxide synthase transcription by cytokines and lipopolysaccharide (LPS) was investigated in lung epithelial adenocarcinoma (A549) cells. LY294002, a dual mTOR and PI3K inhibitor, blocked human inducible nitric oxide synthase (hiNOS) promoter activation and mRNA induction by cytokines and LPS in a PI3K-independent fashion. On gene expression analysis, LY294002 selectively blocked the induction of a subset of 14 LPS/interferon-γ (IFN-γ)-induced genes, previously characterized as signal transducer and activator of transcription-1 (STAT1)-dependent. LY294002, but not wortmannin, inhibited LPS/IFN-γ-dependent STAT1 phosphorylation at Ser-727 and STAT1 activity. Consistent with dual inhibition of mTOR and PI3K by LY294002, dominant-negative mTOR, anti-mTOR small interfering RNA, or rapamycin each inhibited phosphorylation of STAT1 only in the presence of wortmannin. LPS/IFN-γ led to the formation of a macromolecular complex containing mTOR, STAT1, as well as protein kinase Cδ, a known STAT1α kinase. Thus, LPS and IFN-γ activate the PI3K and mTOR pathways, which converge to regulate STAT1-dependent transcription of pro-apoptotic and pro-inflammatory genes in a rapamycin-insensitive manner.

When exposed to cytokines, bacterial products, or viral infection, cells can rapidly initiate the activation of genes encoding proteins that modulate the innate immune response (1). Interferon-γ (IFN-γ), 1 a pro-inflammatory and pro-apoptotic cytokine, regulates host defense, and its activity is modulated by co-stimulation with other cytokines or lipopolysaccharide (LPS) (2–4). The inducible nitric oxide synthase (iNOS) gene is activated in part by IFN-γ, resulting in oxidant-induced cellular damage and control of cell proliferation and apoptosis by newly synthesized nitric oxide (2, 5). We studied the molecular mechanisms leading to the activation of hiNOS transcription as a paradigm to identify intracellular signaling pathways that regulate gene expression in response to inflammatory mediators.

Characterization of the hiNOS promoter structure and function demonstrated that full transcriptional induction by inflammatory mediators required the integration of multiple signaling pathways by the coordinated binding and activation of specific transcription factors. NF-κB, as well as mitogen-activated protein kinase-dependent AP-1, activation and binding were required for full stimulation of the hiNOS promoter in response to cytokines and LPS (6, 7). Optimal hiNOS transcription also required activation by IFN-γ of the pro-apoptotic and pro-inflammatory transcription factor signal transducer and activator of transcription-1 (STAT1) (8). IFN-γ-stimulated phosphorylation of STAT1α at Tyr-701 by Janus kinase led to SH2 domain-dependent dimerization, and was required for nuclear translocation (9). Unlike Tyr-701, phosphorylation at Ser-727 was required for full STAT1 transcriptional activity, but not dimerization or nuclear translocation (9). The signaling events that lead to the activation of STAT1-dependent genes via phosphorylation at Ser-727 are thought to be mediated by mitogen-activated protein kinases, calmodulin-dependent kinase II, or atypical protein kinase C isoforms (PKCδ or ε) (10–12).

In a pharmacological screen to evaluate the role of the phosphatidylinositol 3-kinase (PI3K) pathway in control of hiNOS transcription, the inhibitor LY294002, but not wortmannin, blocked hiNOS promoter activation in response to LPS and IFN-γ, suggesting the involvement of a PI3K-independent target for LY294002. Because LY294002 was also a potent inhibitor of mTOR (13), and mTOR regulated phosphorylation of STAT3 at Ser-727 (14), we hypothesized that mTOR played a role in gene induction through phosphorylation of STAT1 at Ser-727.

mTOR is a protein kinase that regulates cell growth and protein synthesis in response to mitogens and nutrients by activating p70 S6 kinase and 4EBP1 (15). Although many cellular effects of mTOR were discovered by virtue of its sensitivity to rapamycin, a TOR-containing complex was recently described in yeast, the function of which was unaffected by

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§ The abbreviations used are: IFN-γ, interferon-γ; LPS, lipopolysaccharide; CM, cytokine mixture; NOS, nitric oxide synthase; hiNOS, human inducible nitric oxide synthase; TSC, tuberous sclerosis complex; PKB, protein kinase B; PKC, protein kinase C; SH2, Src homology 2; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; STAT1, signal transducer and activator of transcription-1; siRNA, small interfering RNA; ANOVA, analysis of variance.
rapamycin (16). Here, in mammalian cells exposed to inflammatory cytokines, we demonstrate that mTOR converges with the PI3K pathway to activate STAT1 via phosphorylation at Ser-727. The activation of STAT1 is regulated by mTOR kinase activity independent of p70 S6 kinase, is rapamycin-insensitive, and coincides with the formation of a STAT1-mTOR macromolecular complex that also includes PKCδ. These data establish a novel role for mTOR in the control of transcription via activation of STAT1 that is independent of the p70 S6 kinase pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Cytokine Induction—A549 cells (American Type Culture Collection (ATCC) CCL 185) were cultured and stimulated with cytokine mixture (CM) containing IFN-γ, 100 units/ml, interleukin-1β, 0.5 ng/ml, and tumor necrosis factor-α, 10 ng/ml (all from Roche Applied Science), or a mixture of LPS, 100 μg/ml (Sigma), and IFN-γ, 10 units/ml as previously described (6). Rapamycin, LY294002, and/or wortmannin (Biomol or Calbiochem) dissolved in Me2SO were added directly to the cells as indicated.

Transient Transfection and Determination of hiNOS Promoter or STAT1 Reporter Activity—A549 cells were transfected using LipofectAMINE reagent (Invitrogen), 16 μg/ml, with luciferase reporter or mammalian expression vector, 1 μg/ml. Experiments were performed after 36 h, followed by measurement of luciferase activity (Dual Luciferase Assay kit, Promega) or protein immunodetection. In experiments involving transfection with STAT1 reporter vector or cotransfection with pUSE-amp-pyr110, 25 ng/ml pRL-TK (thymidine kinase-driven Renilla luciferase reporter, Promega) was included in the transfection mixture, and promoter activity was normalized to Renilla luciferase activity.

Synthesis and Transfection of Small Interfering RNAs (siRNAs)—The following DNA oligonucleotide templates were synthesized (Invitrogen) for use in a T7 polymerase-based in vitro transcription reaction (Silencer siRNA construction kit, Ambion) to generate double-stranded 21-bp RNA with dideoxythymidine overhangs complementary to a 21-bp region in the human mTOR mRNA: antisense, 5′-AACTCC-GAGAGATGAGTCAAGCCTGTCTC-3′; sense, 5′-AACCTTAGCTACCTC-TCCTGGAGCTGTC-3′. The sequence was not present in any other known mRNA by BLAST analysis. siRNA that was not complementary to any mRNA sequence by BLAST analysis (Ambion) was used as a negative control. A549 cells were plated at a density of 80,000 cells/ml and transfected after 24 h without or with 10 pmol siRNA and 5 μl LipofectAMINE 2000 as per the manufacturer’s protocol (Invitrogen). After 36 h, experiments were performed as indicated.

Plasmids and Antibodies—The plasmid containing the 8.3-kb human iNOS promoter linked to luciferase cDNA (PGL3-biNOS) has been described and characterized previously (9, 17), as were pcDNA1.1 mammalian expression vectors containing hemagglutinin-tagged wild-type and dominant negative/rapamycin-resistant (N2343K/S2035T) mTOR, mTOR dominant negative, and coincides with the formation of a STAT1 activity independent of p70 S6 kinase, is rapamycin-insensitive, and coincides with the formation of a STAT1-mTOR macromolecular complex that also includes PKCδ. These data establish a novel role for mTOR in the control of transcription via activation of STAT1 that is independent of the p70 S6 kinase pathway.

Probe Synthesis for Northern Blotting—The human iNOS cDNA probe was generated by polymerase chain reaction using a human lung cDNA library as template. The oligonucleotide primers corresponding to the hiNOS gene boundary sequences used to generate double-stranded DNA probes were as follows: hiNOS-sense (2125–2144), 5′-TACAGGCTGCTGGAGGCTC-3′; and hiNOS-antisense (2356–2375), 5′-CCATCGGTCCTCAGGACTC-3′. Probes labeled with [32P]dCTP (Rad Prime kit, Gibco) were purified and denatured before addition to hybridization buffer to a final concentration of 2 × 106 cpm/ml.

Isolation of RNA and Northern Blot—Samples (10 μg/lane) of total RNA isolated using Trizol reagent (Invitrogen) were subjected to agarose gel electrophoresis. Loading consistency was assessed by examination of ethidium bromide-stained ribosomal bands. RNA was transferred to nylon membranes (BrightStar Plus, Ambion), pre-hybridized for 1 h, and hybridized (overnight at 42 °C) with [32P]-labeled oligonucleotide probes specific for hiNOS. Membranes were washed and exposed to x-ray film at −70 °C. All procedures were carried out using reagents from Ambion according to the manufacturer’s protocol.

Immunoprecipitation—After incubation, A549 cells were washed once with cold phosphate-buffered saline and lysed in homogenization buffer. Lysates were centrifuged for 5 min, 1000 × g and supernatants were centrifuged for 1 h at 100,000 × g. After centrifuging lysates with 5 μg normal IgG and 20 μl of protein G-Sepharose (Pierce) for 1 h, samples (500 μg) of proteins from high-speed supernatants were incubated with 5 μg of antibody overnight at 4 °C. Complexes were captured with 20 μl protein G-Sepharose and centrifuged at 500 × g for 5 min. Pellets were washed twice with 0.1% Nonidet P-40 in phosphate-buffered saline and boiled in sample buffer for 5 min. Equal volumes of supernatants were loaded in sample buffer for 5 min. Western blots were performed as indicated.

Microarray Analysis—RNA from A549 cells was isolated using Trizol reagent (Invitrogen). For each experimental condition, biotin-labeled RNA probe was synthesized from a double-stranded cDNA template generated from 5 μg of total RNA using protocols and reagents outlined in the manufacturer’s protocol (Affymetrix). Integrity and purity of the RNA and cDNA were assessed by gel electrophoresis and UV absorbance. Microarray chips (Hu95aVer2, Affymetrix) were incubated with fragmented RNA probe, washed, and read using a laser scanner (Hewlett Packard). Images were processed using Micro Array Suite software (Affymetrix). The results reflect duplicate samples analyzed using Genespring 4.2.1 software (Silicon Genetics). The 50th percentile of all measurements was used as a positive control for each sample. Each measurement for each gene was divided by this calculated positive control. The measurement for each gene in each sample was divided by the corresponding value in the control samples.

RESULTS

Inhibition of a LY294002-sensitive Pathway Reveals a PI3K-independent Mechanism for Activation of hiNOS Transcription—To investigate the role of mTOR and PI3K in regulation of the hiNOS promoter, pharmacological, and molecular inhibitors were employed. LY294002, but not wortmannin, inhibited hiNOS promoter activation in response to cytokine mixture (CM: tumor necrosis factor-α, interleukin-1β, IFN-γ) or LPS/IFN-γ (Fig. 1A). Previous studies (19, 20) had demonstrated that inhibition of PI3K by wortmannin potentiated iNOS transcriptional activation, suggesting that LY294002 might inhibit hiNOS transcription via a PI3K-independent mechanism. Consistent with these reports, overexpression of a constitutively active form of the PI3K catalytic unit (p110 CA) suppressed hiNOS transcription via a PI3K-independent mechanism (Fig. 1B).
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**Fig. 1. Different effects of wortmannin and LY294002 on CM- and LPS/IFN-γ-stimulated hiNOS transcriptional activation.** A, A549 cells transiently transfected with PGL3-hiNOS were incubated for 1 h with the indicated concentration of wortmannin (circles) or LY294002 (squares), and then for 6 h without or with CM (filled) or LPS/IFN-γ (empty). Fold-induction of hiNOS promoter activity (stimulated divided by unstimulated luciferase activity) is reported as percentage of the relevant control value (no inhibitor). Data are means of values from 2 to 3 experiments (± half the range or S.E.) with assays in triplicate. *, p < 0.05 by Student’s t test versus control. †, p > 0.05. B, A549 cells were transfected with PGL3-hiNOS and mammalian expression vector empty (EV) or with cDNA encoding constitutively active p110α (p110 CA). After 36 h, cells were incubated for 1 h without or with 100 nM LY294002 or 200 nM wortmannin as indicated, followed by incubation without or with CM or LPS/IFN-γ for 6 h. Fold induction of hiNOS promoter activity is reported as the mean of values from 3 experiments with assays in triplicate (± S.E.). C, A549 cells were incubated as in B before isolation of total RNA, and Northern blot analysis. The 28 S ribosomal band was photographed under UV light. Northern blots are representative of two experiments. D, A549 cells were incubated with 200 nx wortmanin or 100 μM LY294002 for 1 h and then without or with CM or LPS/IFN-γ for 30 min before preparation of lysates. Western blots after reaction with anti-PKBα (PKB) or anti-phospho-PKB Ser-473 (p-PKB) antibodies are shown.

Inhibited a rate-limiting step in hiNOS transcriptional activation distinct from PI3K.

**Inhibition of a LY294002-sensitive Pathway Inhibits the Induction of a Discrete Subset of STAT1-dependent Pro-apoptotic Genes**—To define the LY294002-sensitive reaction involved in control of transcription, we sought a PI3K-independent pharmacological target. Gene expression microarray experiments were performed using RNA isolated from cells treated with LY294002 or wortmanin during incubation with LPS/IFN-γ. Of 12,652 probe sets, 40–45% hybridized under any given experimental condition. Using microarray analysis software (GeneSpring ver 4.2.1, Silicon Genetics), the data set was queried for genes whose expression was perturbed by LY294002 or wortmanin in a fashion similar to that of hiNOS (Fig. 2, Pattern A). Of the 234 genes with a correlation coefficient greater than 0.95, 38 were statistically significant by ANOVA (each p < 0.1, Table I). Of these, 3 unique mRNAs hybridized to different probes for the same gene on the array (i.e. Fas, Apo-1, RREST1, SP-100), and sixteen were related to apoptosis and/or growth inhibition. The expression of 3 genes representing the proximal portion of the FasL-stimulated apoptosis pathway was co-regulated, and included Fas, Caspase-8- and Fas-associated death domain-like apoptosis regulator, and Bcl-2 homology 3-interacting domain death agonist (BID). Ten of these genes were known to be regulated by STAT1, and one, Fas, specifically by phosphorylation at Ser-727 (21).

Although LY294002 potently inhibited mTOR and PI3K (13), wortmanin inhibited the activation of p70 S6 kinase and PI3K independent of mTOR (22). Thus, to identify genes likely to be regulated independent of mTOR, the data set was queried for genes whose expression is inhibited by LY294002 or wortmanin in the presence of LPS/IFN-γ (Fig. 2, Pattern B). Of 108 genes with a correlation coefficient greater than 0.95, 44 exhibited statistically significant expression in at least 1 experimental condition (data not shown). The genes correlating with pattern B were all different from those correlating with pattern A, indicating the absence of overlap between LY294002- and wortmannin-sensitive pathways. Thus, LY294002 unmasks a discrete subset of genes related to apoptosis and STAT1 activation in the presence of LPS/IFN-γ.

**LY294002 Inhibits Activation of STAT1 by Attenuating Phosphorylation at Ser-727—**STAT1 phosphorylation at Ser-727 in response to IFN-γ was enhanced in the presence of LPS (Fig. 3A), consistent with additive agonist effects on intracellular signaling. Phosphorylation of STAT1 at Ser-727 was detected in unstimulated cells and increased over 30 min in response to LPS/IFN-γ, but not wortmanin, inhibited STAT1 phosphorylation at Ser-727 (Fig. 3B), consistent with their effects on hiNOS transcription (Fig. 1A), as well as a role for STAT1 in the activation of hiNOS transcription (8). LY294002 had no effect on STAT1 phospho-Tyr-701 levels, indicating that the LY294002 target is independent of the Janus kinase tyrosine kinase pathway. LPS/IFN-γ-stimulated STAT1 activity was inhibited by LY294002, and not by wortmanin, providing a functional correlate for phosphorylation of STAT1 at Ser-727 (Fig. 3C). Consistent with its effect on mTOR, LY294002 inhibited phosphorylation of p70 S6 kinase at T389 and mTOR at Ser-2481 (autophosphorylation site), both markers of mTOR kinase activity (Fig. 3D) (23, 24).

The P13K pathway can regulate mTOR-dependent signaling via protein kinase B phosphorylation of mTOR at Ser-2448, or through phosphorylation of the mTOR suppressor TSC2 (25–27). However, in A549 cells, neither wortmanin nor LY294002 inhibited phosphorylation of mTOR at Ser-2448, suggesting that direct regulation of mTOR by protein kinase B was un-
likely to affect downstream signaling in response to LPS/IFN-γ. Moreover, wortmannin did not inhibit phosphorylation of mTOR at Ser-2481, confirming a previous study that demonstrated a weak inhibitory effect of wortmannin on mTOR kinase activity (13). Consistent with a previous study (22) demonstrating PI3K regulation of p70 S6 kinase independent of mTOR, wortmannin did not inhibit phosphorylation of p70 S6 kinase at Thr-389 (Fig. 3B). This finding is consistent with the lack of rapamycin abolished phosphorylation of p70 S6 kinase at Thr-389, phosphorylation of STAT1 at Ser-727, cells transfected with empty vector or wild-type mTOR, suggesting that PI3K inhibition did not override mTOR-mediated STAT1 phosphorylation (Fig. 4A, lanes 4 and 8). Overexpression of dominant-negative mTOR increased total STAT1 levels, in part explaining the increase in basal phosphorylation of STAT1 at Ser-727 (4A, lanes 9 and 10). Moreover, although rapamycin abolished phosphorylation of p70 S6 kinase at Thr-389, phosphorylation of STAT1 at Ser-727 in cells overexpressing wild-type mTOR was not affected (data not shown).

To mimic the dual inhibition of mTOR and PI3K by LY29002, we assessed STAT1 phosphorylation in the presence of kinase-dead mTOR and wortmannin. Simultaneous inhibition of PI3K and mTOR decreased phosphorylation of STAT1 at Ser-727, indicating that under conditions of reduced PI3K activity, mTOR is required for LPS/IFN-γ-stimulated STAT1 activation (Fig. 4A, lanes 11 and 12). Wortmannin alone did not attenuate phosphorylation of STAT1 in cells transfected with empty vector or wild-type mTOR, suggesting that PI3K inhibition did not override mTOR-mediated STAT1 phosphorylation (Fig. 4A, lanes 4 and 8). Overexpression of dominant-negative mTOR increased total STAT1 levels, in part explaining the increase in basal phosphorylation of STAT1 at Ser-727 (4A, lanes 9–12). When densitometry was performed, and phosphorylation of STAT1 levels were normalized to total STAT1 levels, the reduction in phosphorylation of STAT1 at Ser-727 due to overexpression of dominant-negative mTOR in the presence of wortmannin was confirmed (Fig. 4B). Moreover, rapamycin and wortmannin had an additive inhibitory effect on STAT1 activation by LPS/IFN-γ (Fig. 4C).

In agreement with the experiments using overexpressed dominant-negative mTOR, transfection of cells with anti-mTOR siRNA led to the inhibition of LPS/IFN-γ-stimulated...
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The inability of mTOR inhibitors to attenuate phosphorylation of STAT1 at Ser-727 suggested that mTOR might not be a direct kinase for STAT1. Previous studies demonstrated a role for mTOR in the activation of PKCδ (28), as well as a role for PKCδ in the phosphorylation of STAT1 at Ser-727 (11). We therefore performed immunoprecipitation experiments to further define components of the mTOR-containing complex. Antibodies raised against STAT1α or PKCδ both immunoprecipitated mTOR, and the respective interactions were more prominent when cells were incubated with LPS/IFN-γ (Fig. 5C, Pel). Similarly, incubation with LPS/IFN-γ increased the interaction between STAT1α and PKCδ, as demonstrated by immunoprecipitations with their respective antibodies (Fig. 5C, Pel). The amounts of PKCδ or STAT1α in the complex represented a small portion of the total cellular PKCδ or STAT1α, respectively (compare lanes 3 and 4 with lanes 5 and 6). In agreement, whereas anti-STAT1α antibody depleted the cell lysates of STAT1α, there was only a small reduction in PKCδ levels (Fig. 5C, Sup). The same was true for the depletion of STAT1α by anti-PKCδ antibody (Fig. 5C, Sup). Consistent with the interaction of mTOR with both PKCδ and STAT1α, antibodies to the latter reduced the amount of mTOR in the lysate. Therefore, in response to LPS/IFN-γ, mTOR regulates STAT1 in a rapamycin-insensitive and p70 S6 kinase-independent fashion through its interaction with PKCδ and STAT1.

**DISCUSSION**

The studies presented here establish a novel role for mTOR in the immediate transcriptional response to inflammatory mediators. Using pharmacological and molecular methods, we demonstrated that mTOR regulates phosphorylation of STAT1 at Ser-727. These conclusions are supported by the finding that mTOR and STAT1 are recruited to a macromolecular complex in the presence of LPS and IFN-γ. The interaction of PKCδ with mTOR and STAT1 in the presence of LPS/IFN-γ (Fig. 5C) supports a dual role for PI3K and mTOR in the activation of STAT1, as PKCδ is a target of both the mTOR and PI3K pathways (28–30), and PKCδ can phosphorylate STAT1 at Ser-727 (11). The mTOR/STAT1 pathway would appear to be distinct from the mTOR/p70 S6 kinase pathway because it is unaffected by rapamycin (Figs. 3C and 4C), or wortmannin (Fig. 3D) alone. Thus, mTOR is a regulator of transcription through its intermolecular interactions with STAT1 and PKCδ.

Gene expression profiling by microarray experiments allowed for the identification of a discrete subset of genes involved in innate immunity that are induced by LPS and IFN-γ, and the transcription of which depended upon the parallel and simultaneous activation of mTOR and PI3K (Fig. 6 and Table I). Of these, the following genes were previously shown to modulate innate immunity in response to IFN-γ: 2-5A synthetase, SP100, promyelocytic leukemia (PML), γ-interferon-inducible lysosomal thiol reductase (GILT), indoleamine 2,3-dioxygenase (IDO), and monocyte chemoattractant protein-1 (MCP-1) (2). Of note, LY294002, a dual mTOR and PI3K inhibitor, attenuated the specific induction of genes in a known pro-apoptotic pathway (i.e. Caspase-8 and Caspase-9-associated death domain-like apoptosis regulator, Caspase-8, and BH3-interacting domain death agonist) by LPS/IFN-γ. Thus, mTOR-dependent activation of STAT1 in cells exposed to LPS/IFN-γ activates a transcriptional program for host defense and cellular sensitization to pro-apoptotic factors.

Although mTOR was sufficient to cause phosphorylation of STAT1 (Fig. 4A), its activity was rate-limiting only under conditions of reduced PI3K activity (Fig. 4). Similarly, previous studies (25, 31, 32) showed that the relative role of TOR proteins in signaling depended on the cellular environment (e.g. nutrient availability, mitogen stimulation, and energy avail-

**Fig. 3.** STAT1 is activated by LPS and IFN-γ and inhibited by LY294002, but not wortmannin. A, A549 cells were treated without or with LPS/IFN-γ for 5, 15, or 30 min after serum starvation for 1 h. In separate experiments, cells were incubated in serum-free medium, without or with LPS, 100 μg/ml IFN-γ, 100 units/ml or both for 30 min after serum starvation for 1 h. Phosphorylation of STAT1 at Ser-727 was detected by Western blot analysis. B, A549 cells were incubated with 200 nM wortmannin or 100 μM LY294002 for 1 h and then without or with LPS/IFN-γ for 30 min before preparation of lysates. Western blots after reaction with anti-phospho-STAT1Ser-727, anti-phospho-STAT1 Tyr-701, or anti-STAT1 antibodies are shown. Data are representative of 3 experiments; each blot shown is from the same experiment. C, A549 cells transiently transfected with STAT1 reporter vector (GAS-Luc) were incubated for 1 h without or with 100 μM LY294002, 200 nM wortmannin, or rapamycin, 50 ng/ml, then without or with LPS/IFN-γ for 6 h as indicated, before assay of cell lysates for luciferase activity. STAT1-stimulated luciferase activity was normalized to Renilla luciferase activity; data are representative of 3 experiments with assays in triplicate (± S.E.). D, A549 cells were incubated as in B. Western blots after reaction with anti-phospho-p70 S6 kinase Thr-389, anti-phospho-mTOR Ser-2481 or Ser-2448, or anti-mTOR antibodies are shown. Data are representative of 3 experiments; each blot shown is from the same experiment.
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Wild-type (WT) A549 cells were transfected with empty vector control (EV), mammalian expression vector containing cDNA encoding hemagglutinin-tagged A549 cells were transfected with EV, WT, or NK. A549 cells were transfected with EV, WT, or NK. A549 cells were transfected with empty vector control (EV), mammalian expression vector containing cDNA encoding hemagglutinin-tagged A549 cells were transfected with EV, WT, or NK. A549 cells were transfected with empty vector control (EV), mammalian expression vector containing cDNA encoding hemagglutinin-tagged A549 cells were transfected with EV, WT, or NK.

Fig. 4. mTOR kinase activity regulates activation of STAT1. A, A549 cells were transfected with empty vector control (EV), mammalian expression vector containing cDNA encoding hemagglutinin-tagged wild-type (WT), or that containing kinase dead/rapamycin resistant (NK) mTOR. After 36 h, cells were incubated without or with 50 nM wortmannin before treatment without or with LPS/IFN-γ for 30 min. Cell lysates were analyzed by Western blot with anti-phospho-STAT1 Ser-727, anti-STAT1α, or anti-hemagglutinin antibodies. Blots shown are representative of 3 experiments and are from the same experiment number from 1 to 12. B, bands corresponding to phospho-STAT1 and STAT1α were measured by densitometry. Mean fold increases in the ratio of phospho-STAT1 to total STAT1 band density from 3 separate experiments (± S.E.) are shown. C, A549 cells were transiently transfected and treated without or with inhibitors or LPS/IFN-γ for 30 min. Cell lysates were analyzed by Western blot with anti-phospho-STAT1 Ser-727, anti-STAT1α, or anti-hemagglutinin antibodies. Blots shown are representative of 3 experiments and are from the same experiment number from 1 to 12. B, bands corresponding to phospho-STAT1 and STAT1α were measured by densitometry. Mean fold increases in the ratio of phospho-STAT1 to total STAT1 band density from 3 separate experiments (± S.E.) are shown. D, A549 cells were transfected with control or anti-mTOR siRNA for 36 h. After incubation without or with 50 nM wortmannin in serum-free medium, cells were treated without or with LPS/IFN-γ for 30 min before lysis of the cells, and Western blotting for the indicated proteins. Each blot shown is from the same experiment, and representative of three experiments.
The regulation of mTOR has been of particular interest with regard to the pathogenesis of tuberous sclerosis complex (TSC), a disease inherited in autosomal dominant fashion, and caused by defects in the TSC1 or TSC2 genes (47). Defects in the TSC genes are also implicated in the pathogenesis of lymphangioleiomyomatosis (LAM), a disease of abnormal smooth muscle cell proliferation that may occur sporadically or in patients with TSC (47). The TSC2 gene product, tuberin, was phosphorylated at specific residues by protein kinase B (27). Inhibition of PI3K-dependent PKB activity led to a decrease in mTOR function, as assessed by p70 S6 kinase phosphorylation or activity, suggesting that TSC2 is a suppressor of mTOR, and thus a negative regulator of cell growth (26). Similarly, during LPS/IFN-γ stimulation, there was a reduction in mTOR auto-phosphorylation, or p70 S6 kinase phosphorylation (Fig. 3 D), in the presence of wortmannin. However, in the absence of rapamycin or kinase-deficient mTOR, wortmannin did not inhibit STAT1 phosphorylation or activity (Fig. 4). Therefore, LPS/IFN-γ-dependent regulation of STAT1 through mTOR appears to be distinct from that of p70 S6 kinase through TSC2 and mTOR. Whereas the mTOR/p70 S6 kinase pathway stimulates cell growth, and is rapamycin-sensitive, the mTOR/STAT1 pathway activates a discrete set of pro-inflammatory and pro-apoptotic genes, and in the absence of PI3K inhibition is rapamycin-insensitive.

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