

# The TSC-mTOR Signaling Pathway Regulates the Innate Inflammatory Response

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## SUMMARY

The innate inflammatory immune response must be tightly controlled to avoid damage to the host. Here, we showed that the tuberous sclerosis complex-mammalian target of rapamycin (TSC-mTOR) pathway regulated inflammatory responses after bacterial stimulation in monocytes, macrophages, and primary dendritic cells. Inhibition of mTOR by rapamycin promoted production of proinflammatory cytokines via the transcription factor NF- $\kappa$ B but blocked the release of interleukin-10 via the transcription factor STAT3. Conversely, deletion of TSC2, the key negative regulator of mTOR, diminished NF- $\kappa$ B but enhanced STAT3 activity and reversed this proinflammatory cytokine shift. Rapamycin-hyperactivated monocytes displayed a strong T helper 1 (Th1) cell- and Th17 cell-polarizing potency. Inhibition of mTOR in vivo regulated the inflammatory response and protected genetically susceptible mice against lethal *Listeria monocytogenes* infection. These data identify the TSC2-mTOR pathway as a key regulator of innate immune homeostasis with broad clinical implications for infectious and autoimmune diseases, vaccination, cancer, and transplantation.

## INTRODUCTION

Inflammation, an essential defense mechanism, is a key event in the response to bacterial and viral infections (Kimbrell and Beutler, 2001). The coordinated secretion of pro- and anti-inflammatory cytokines such as IL-12 and IL-10, respectively, by myeloid mononuclear phagocytes including monocytes, macrophages, and dendritic cells (DCs) is indispensable for effective immunity (Trinchieri, 2003). Nevertheless, the cellular mechanisms regulating pro- versus anti-inflammatory responses are

poorly defined. Phosphatidylinositol 3-kinase (PI3K), which is activated early after bacterial stimulation, was elucidated as an important molecule in innate immune cells that regulates IL-10 and IL-12 production. Genetic or pharmacological ablation of PI3K in monocytes or peripheral DCs inhibits IL-10 and boosts IL-12 production (Fukao et al., 2002; Martin et al., 2003). However, the molecular mechanisms that control inflammatory responses via PI3K signal transduction in myeloid cells are unknown.

The tuberous sclerosis complex (TSC) protein 2 belongs to a group of proteins linked to the PI3K pathway (Wullschlegler et al., 2006). TSC2 is a tumor suppressor that forms a heterodimeric complex with TSC1. Mutations in TSC1 or TSC2 give rise to the hamartoma-syndrome tuberous sclerosis complex and the proliferative lung disorder lymphangioleiomyomatosis (Paul and Thiele, 2008). TSC2 is phosphorylated and inactivated by the protein kinase Akt, which itself is activated by PI3K (Inoki et al., 2002). Importantly, the TSC1-TSC2 complex negatively regulates the mammalian target of rapamycin (mTOR) (Inoki et al., 2002). mTOR, an essential serine-threonine kinase, is a central regulator of cell growth and proliferation. mTOR directly phosphorylates the ribosomal p70S6 kinase (p70S6K) and the initiation factor 4E-binding protein 1 (4E-BP1) to initiate translation of distinct mRNAs (Hay and Sonenberg, 2004). The prototypic mTOR inhibitor rapamycin, a bacterial macrolide with potent immunosuppressive and antitumor activities, is also currently evaluated to treat tuberous sclerosis and lymphangioleiomyomatosis (Paul and Thiele, 2008). Because of the exquisite sensitivity of T cells to the cell-cycle-blocking effects of rapamycin, mTOR inhibition was introduced in clinical transplantation (Huang et al., 2003). Interestingly, the increased usage of rapamycin has been accompanied by distinct inflammatory events including lymphocytic alveolitis, interstitial pneumonitis, and severe forms of glomerulonephritis (Dittrich et al., 2004; Izzedine et al., 2005; Singer et al., 2000; Thauan et al., 2005). However, the underlying mechanisms of these proinflammatory effects are largely unknown.

Some investigators reported an anti-inflammatory property of rapamycin on in vitro-generated DCs, whereas in

in vitro-generated macrophages, rapamycin seems to enhance IL-23 production (Monti et al., 2003; Taner et al., 2005; Yang et al., 2006). Here, we investigated whether TSC and mTOR are involved in the regulation of inflammatory mediators upon bacterial stimulation of freshly isolated untouched mononuclear phagocytes. We showed that activation of the mTOR pathway limits inflammatory responses by blocking NF- $\kappa$ B and enhancing STAT3 activity.

## RESULTS

### Rapamycin Enhances Proinflammatory Cytokine Production

To define a potential role of mTOR in the regulation of pro- versus anti-inflammatory cytokines, we first assessed the effects of prototypic bacterial stimuli, such as lipopolysaccharide (LPS) or *Staphylococcus aureus* cells (SACs), on human peripheral blood mononuclear cells (PBMCs) in the presence or absence of rapamycin. mTOR inhibition by rapamycin in PBMCs stimulated with LPS or SACs led to a potent upregulation of IL-12p40 production (Figures 1A and 1B). Concurrently, the anti-inflammatory cytokine IL-10 was profoundly suppressed (Figure 1A). Because monocytes and myeloid DCs are the main producers of IL-12 in the blood (Trinchieri, 2003), we next investigated the effects of rapamycin on freshly isolated LPS- or SAC-stimulated monocytes and obtained similar results, even at low-nanomolar concentrations of the mTOR inhibitor (Figures 1C and 1D). Activation of monocytes with the intracellular human pathogen *Listeria monocytogenes* (*L.m.*), Pam3Cys, or flagellin likewise promoted IL-12p40 and inhibited IL-10 production after rapamycin pretreatment (Figure 1E and data not shown). Importantly, mTOR inhibition increased the production of the biologically active heterodimers IL-12p70 and IL-23 (Figures 1F and 1G), which share the IL-12p40 subunit, and of the proinflammatory cytokines TNF- $\alpha$  and IL-6 (Figures 1H and 1I and Figure S1 available online). When freshly isolated primary myeloid DC were tested (Figure S2), a similar increase in LPS- and SAC-mediated IL-12p40 production and decrease in IL-10 production was observed upon treatment with rapamycin (Figures 1J and 1K). These results indicate that mTOR inhibition alters the cytokine balance of monocytes and myeloid DC toward a proinflammatory phenotype upon microbial stimulation.

### Bacterial Stimuli Activate the mTOR Pathway Downstream of PI3K

The ability of rapamycin to affect cytokine production suggested an involvement of the mTOR signaling pathway in the coordinated production of pro- versus anti-inflammatory cytokines. However, the mTOR pathway has not been defined in mononuclear phagocytes, but it is known that bacterial stimuli activate PI3K, which leads to phosphorylation of Akt (Arbibe et al., 2000). In other cell types, it has been further shown that activation of Akt promotes the phosphorylation and inactivation of TSC2 (Manning et al., 2002). We found that LPS induced Akt-dependent phosphorylation of TSC2 on Ser939 in human monocytes (Figure 2A). Moreover, LPS also activated the downstream mediators of mTOR, 4E-BP1, p70S6K, and S6, whereas rapamycin inhibited their phosphorylation (Figures 2A and 2B). In addition, blockade of Akt inhibited phosphorylation of 4E-BP1 and S6, confirming that the mTOR pathway is downstream of Akt in

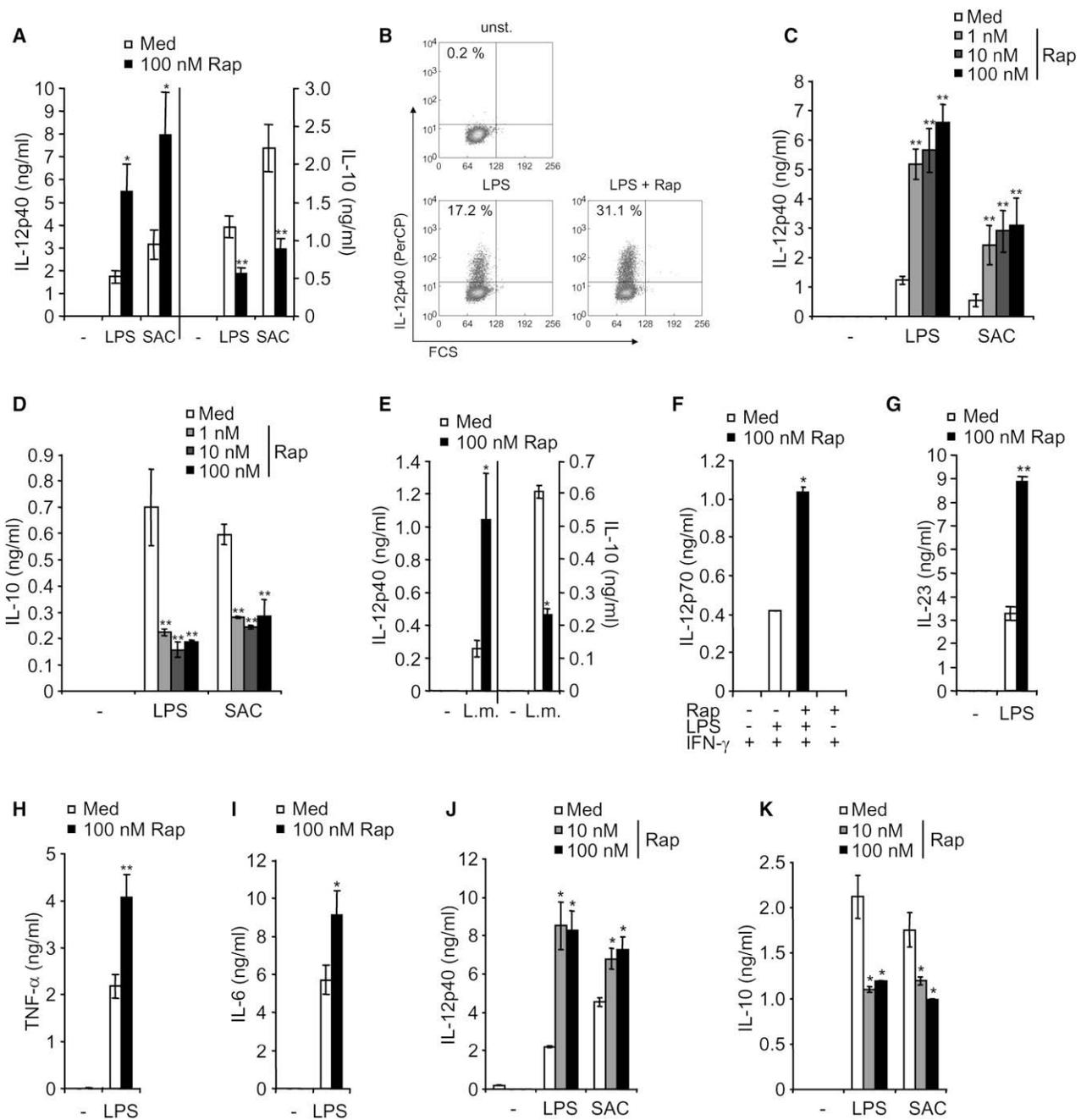
monocytes (Figure 2C and Figure S3). Murine bone-marrow-derived macrophages (BMDMs) similarly activated the mTOR pathway after in vitro infection with live *L.m.* and rapamycin inhibited this activation (Figure 2D).

Ser2448 is a well-known phosphorylation site of mTOR and sensitive to PI3K inhibition (Sekulic et al., 2000). Ser2448 phosphorylation of mTOR was evident in LPS-stimulated monocytes (Figure 2E) and could be prevented by pharmacological inhibition of PI3K with wortmannin (Figure 2E), demonstrating that PI3K signaling is upstream of mTOR in human monocytes. Accordingly, PI3K inhibition augmented IL-12 but suppressed IL-10 production (Figure 2F). Taken together, these results provide evidence that the mTOR pathway is downstream of PI3K and that this pathway can be activated by bacterial stimuli in human and murine myeloid cells.

### The TSC2-mTOR Pathway Mediates the Effects of Rapamycin on Cytokine Production

Rapamycin associates with FK506-binding protein 12 (FKBP12) to selectively bind and inhibit a complex consisting of mTOR, mLST8, and Raptor (mTORC1) but not a complex of mTOR, mLST8, and Rictor (mTORC2) (Wullschlegel et al., 2006). However, prolonged treatment with rapamycin has been shown to inhibit mTORC2 signaling in distinct cell lines (Rosner and Hengstschlager, 2008; Sarbassov et al., 2006). Pre-exposure of monocytes to rapamycin and subsequent washout of the inhibitor similarly deviated cytokine production after LPS stimulation indicating mTORC1-specific effects (Figure 3A). Similar to rapamycin, the immunosuppressive macrolide FK506 engages FKBP12 and blocks calcineurin phosphatase instead of mTOR. We observed that FK506 did not significantly modulate IL-12 or IL-10 production (Figure 3B) but was able to outcompete the rapamycin effects (Figure 3C). Because rapamycin regulates the balance of IL-12 and IL-10, hyperactivation of mTOR after bacterial stimulation ought to reverse the pattern of cytokine production. Accordingly, amino acids or the nucleotide ATP, which have been shown to activate mTOR signaling (Yang and Guan, 2007), but not UTP inhibited IL-12 and simultaneously enhanced IL-10 production (Figures 3D and 3E).

To directly address whether the mTOR pathway controls inflammatory cytokine production, we made use of murine embryonic fibroblasts (MEFs) lacking TSC2, a key negative regulator of mTOR. TSC2 deficiency led to constitutive activation of the mTOR pathway (Figure 4A). We next examined whether loss of TSC2 might affect cytokine production in a reciprocal manner to rapamycin. Because MEF do not produce IL-12p40, we transfected MEF with an IL-12p40 promoter plasmid to assess IL-12p40 induction. Indeed, IL-12p40 was inhibited markedly in *Tsc2*<sup>-/-</sup> cells and could not be induced by LPS stimulation (Figure 4B), whereas IL-10 was increased in *Tsc2*<sup>-/-</sup> cells (Figure 4C). Moreover, IL-6 production was partly inhibited in *Tsc2*<sup>-/-</sup> cells (Figure 4D). To confirm the involvement of TSC2 also in myeloid immune cells, we employed RNA interference in THP-1 cells to knock down TSC2 (Figure 4E). IL-12p40 and TNF- $\alpha$  production was reduced in TSC2-silenced cells upon LPS stimulation, whereas IL-10 was increased (Figures 4F, 4G, and 4H). In addition to the data obtained with the mTOR inhibitor rapamycin, these results demonstrate that the TSC2-mTOR pathway critically controls inflammatory cytokine production.



**Figure 1. Rapamycin Differentially Modulates Pro- and Anti-inflammatory Cytokine Production**

(A and B) Human PBMC were preincubated with rapamycin or medium and then stimulated with LPS or SAC. Production of IL-12p40 and IL-10 was determined by (A) ELISA or (B) intracellular cytokine staining. Bars represent the mean ± SEM of five donors.

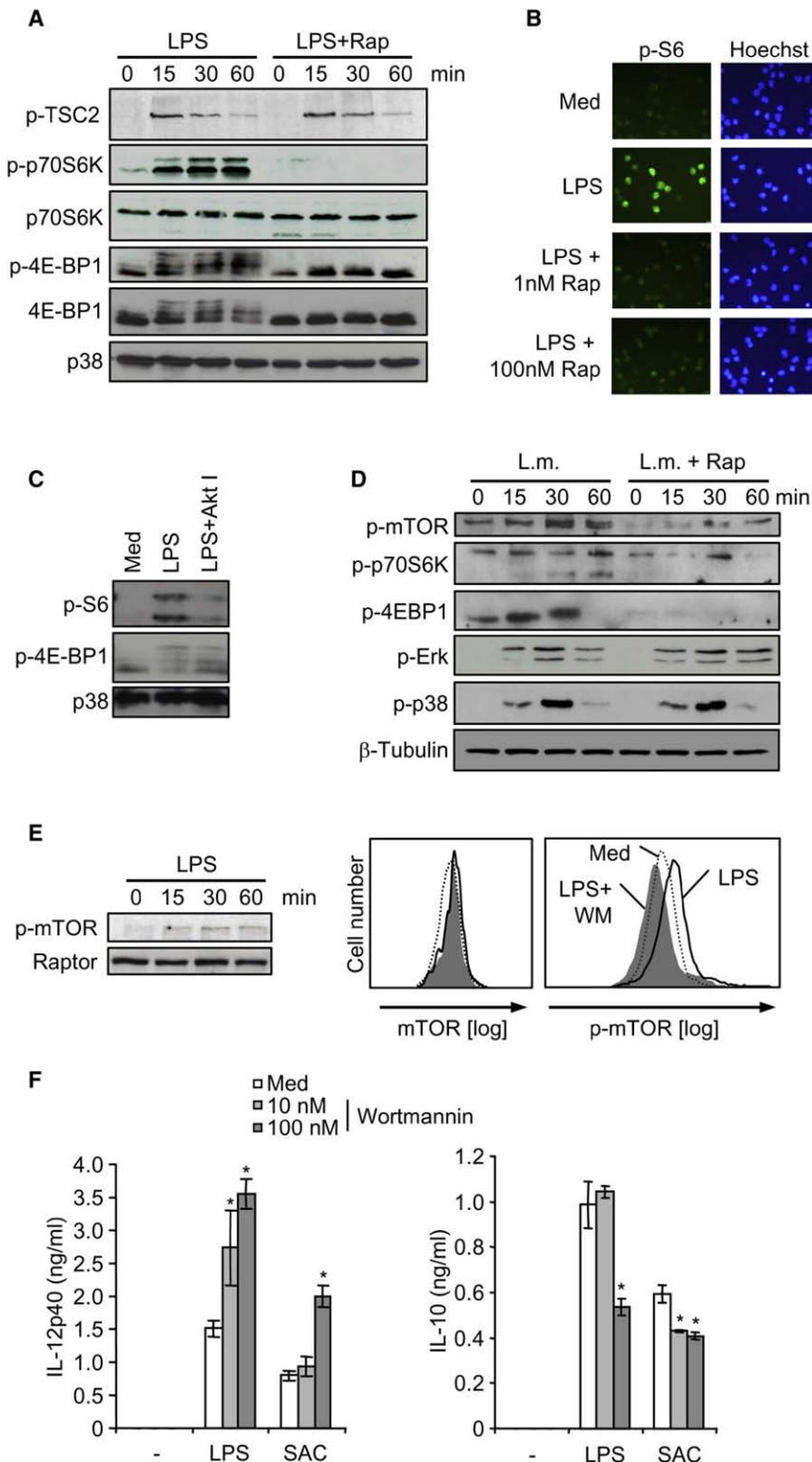
(C–I) Human monocytes were pretreated with medium or rapamycin and then stimulated with medium, LPS, SAC, *L.m.*, or LPS and IFN-γ as indicated. IL-12p40 (C and E), IL-10 (D and E), IL-12p70 (F), IL-23 (G), TNF-α (H), and IL-6 (I) in the supernatants were determined by ELISA (mean ± SEM of three [C–D] or nine [H and I] donors).

(J and K) Myeloid DCs were stimulated as indicated. IL-12p40 (J) and IL-10 (K) in the supernatants were determined by ELISA. Data are representative of three separate experiments (mean ± SD). \*p < 0.05, \*\*p < 0.01 compared with the respective LPS or SAC controls.

**mTOR Negatively Regulates NF-κB Activation**

We went on to elucidate the downstream signals of mTOR, which regulate the IL-12 and IL-10 balance. IL-10 is a well-known endogenous inhibitor of IL-12 production. However, the exogenous addition of IL-10 or neutralizing anti-IL-10 did not change

the effect of rapamycin on IL-12p40 production, indicating an IL-10-independent upregulation of IL-12p40 by mTOR inhibition (Figure S4). Moreover, the protein-synthesis inhibitor cycloheximide did not reverse the rapamycin-induced cytokine alteration, indicating that new protein synthesis is not required for the



**Figure 2. Bacterial Stimuli Activate the mTOR Pathway**

(A–C) Human monocytes were preincubated with rapamycin (Rap), 500 nM Akt inhibitor IV (Akt I), or medium as indicated and stimulated with LPS. As shown in (A) and (C), whole-cell lysates were analyzed by immunoblotting. As shown in (B), phosphorylation of S6 at 30 min after stimulation was detected by immunofluorescence.

(D) BMDMs were treated as indicated and then stimulated with  $10^7$  live *L.m.* Immunoblotting was performed with the indicated antibodies.

(E) Monocytes were preincubated with 100 nM wortmannin (WM) or medium and then stimulated with LPS for 30 min as indicated. p-mTOR was assessed by immunoblotting and intracellular staining. Total Raptor or mTOR protein served as the control.

(F) Human monocytes were treated as indicated. Production of IL-12p40 and IL-10 was determined by ELISA. Data are representative of three separate experiments (mean  $\pm$  SD). \* $p < 0.05$  compared with the respective LPS or SAC controls.

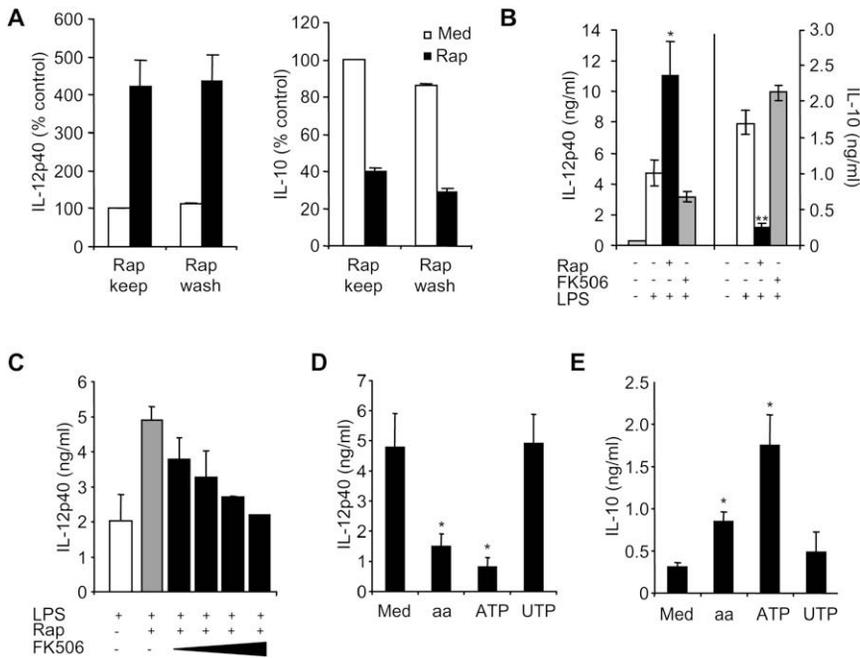
rapamycin enhanced JNK activity (Figure 5A), the main target of JNK, the transcription factor complex AP-1, was not active in monocytes, as demonstrated by EMSA and luciferase reporter studies (Figure 5C and Figure S6). These findings suggest a distinct mTOR-sensitive pathway.

The transcription factor NF- $\kappa$ B is a master regulator of proinflammatory responses and a major regulator of IL-12p40. We observed enhanced NF- $\kappa$ B activation in primary human monocytes, when they were treated with rapamycin and LPS compared to LPS alone, indicating that mTOR negatively regulates NF- $\kappa$ B (Figure 5B). Luciferase reporter assays in THP-1 and U937 cells confirmed this hyperactivation of NF- $\kappa$ B, but not of CRE or AP-1, after mTOR inhibition (Figure 5C and Figure S7). In line with these results, rapamycin not only enhanced but also prolonged IL-12p40 and TNF- $\alpha$  production in monocytes for up to 20 hr after LPS stimulation (Figure S8).

Because the mTOR pathway is constitutively active in *Tsc2*<sup>-/-</sup> cells (Figure 4A), these cells should have a decreased ability to activate NF- $\kappa$ B. Accordingly, *Tsc2*<sup>-/-</sup> cells displayed a significantly reduced LPS-inducible and basal NF- $\kappa$ B

proinflammatory phenotype of mTOR-inhibited cells (Figure S5). Next, we examined the activation of the kinases p38, ERK, and JNK, which are well-known regulators of IL-12 expression (Dong et al., 2002). Phosphorylation of p38 and ERK was not influenced by rapamycin (Figure 2D and Figure 5A). Although rap-

activity (Figure 5D). Noticeably, rapamycin rescued NF- $\kappa$ B activity in LPS-triggered *Tsc2*<sup>-/-</sup> cells to a significant extent (Figure 5D). In agreement, nuclear translocation of NF- $\kappa$ B was reduced in *Tsc2*<sup>-/-</sup> cells (Figure 5E). Supershift assays revealed that the composition of NF- $\kappa$ B family members on the NF- $\kappa$ B



**Figure 3. mTOR Controls Pro- versus Anti-inflammatory Cytokine Production**

(A) Human monocytes were pretreated with medium or rapamycin for 60 min, cells were washed as indicated with medium and stimulated with LPS. Production of IL-12p40 and IL-10 was determined by ELISA and is depicted as the percentage  $\pm$  SEM of the LPS-stimulated control ( $n=3$ ). (B) Monocytes were treated as indicated. Production of IL-12p40 and IL-10 was determined by ELISA. Data are representative of three separate experiments (mean  $\pm$  SD).

(C) Monocytes were treated with rapamycin with or without increasing doses of FK506 (1, 10, 100, and 500 nM) and then stimulated with LPS and assayed for IL-12p40 ( $n=2$ ).

(D and E) Human monocytes, cultured in serum-free medium, were incubated with amino acids (aa), ATP, or UTP and then stimulated with SAC. Cell-free supernatants were analyzed for IL-12p40 and IL-10 (mean  $\pm$  SEM;  $n=3$ ). \* $p < 0.05$ , \*\* $p < 0.01$  compared with the respective LPS controls.

promoter was not altered by rapamycin, and p65 was mainly occupying the NF- $\kappa$ B binding site (Figure S9), suggesting that mTOR might negatively modulate p65 activity to dampen NF- $\kappa$ B activation. The transactivation domain (TAD) of p65 recruits cofactors to enhance transcription (Perkins, 2007). Moreover, PTEN and PI3K might negatively control NF- $\kappa$ B activity through p65(TAD) (Mayo et al., 2002). To test whether mTOR controls the activity of p65(TAD), we utilized a p65(TAD)-Gal4 fusion protein in luciferase reporter assays (Rocha et al., 2003). p65(TAD) was hyperactivated when mTOR was inhibited in human THP-1 and murine MEF cells (Figures 5F and 5G). Moreover, p65(TAD) activity was not induced by LPS in *Tsc2*<sup>-/-</sup> cells, whereas rapamycin restored LPS-dependent p65(TAD) activity in these cells (Figure 5G).

To directly show that the enhanced NF- $\kappa$ B activity is responsible for the observed effects of mTOR inhibition, we blocked NF- $\kappa$ B signaling with SN50, a cell-permeable peptide, which inhibits NF- $\kappa$ B translocation to the nucleus (Lin et al., 1995). SN50 abolished the rapamycin-induced augmentation of IL-12p40 and TNF- $\alpha$  production in human monocytes (Figures 5H and 5I). Collectively, these results demonstrate that the mTOR pathway limits NF- $\kappa$ B activity in myeloid cells by modulation of the p65 transactivation domain to regulate proinflammatory cytokine production.

### mTOR Controls STAT3 Activation

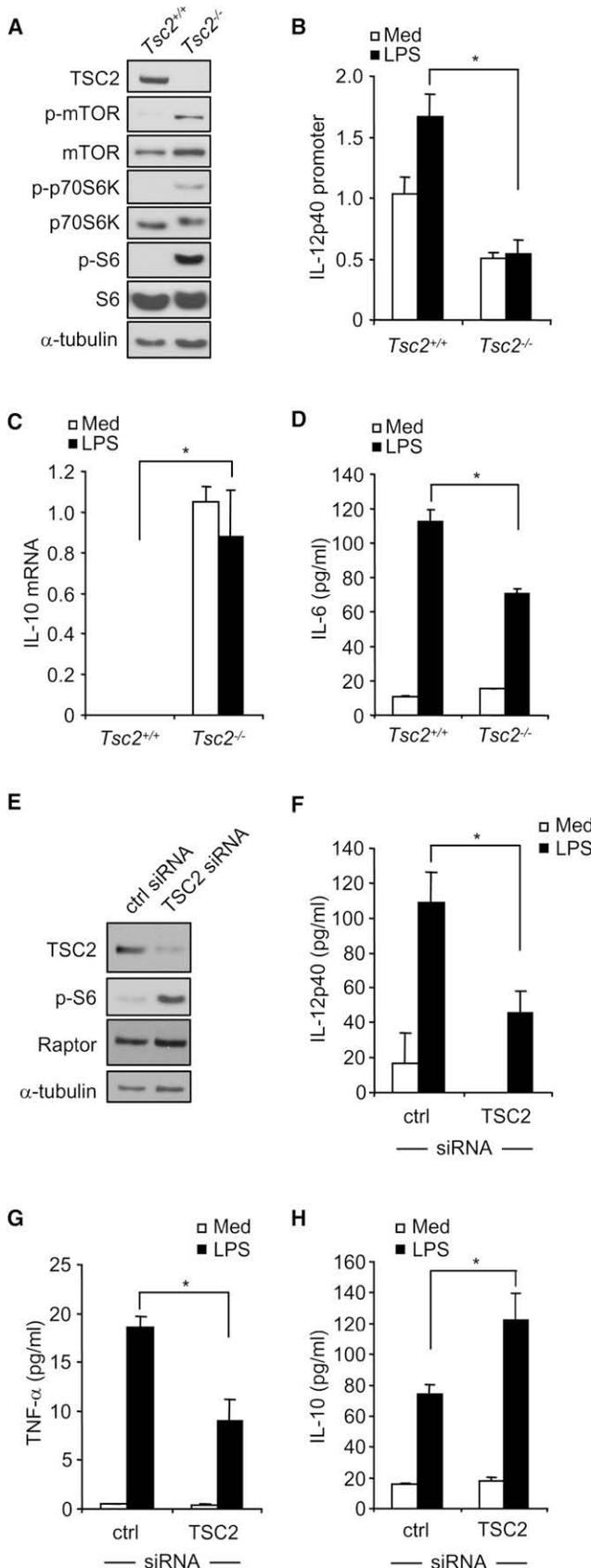
Because NF- $\kappa$ B hyperactivation cannot explain IL-10 blockade induced by rapamycin, we investigated the effects of rapamycin on STAT3, given that STAT3 is a major transcription factor for IL-10 in human monocytes (Benkhart et al., 2000; Cheng et al., 2003) and mTOR has been implicated in the regulation of STAT3 (Karras et al., 1997). Indeed, rapamycin inhibited tyrosine phosphorylation of STAT3 and reduced STAT3 activity (Figure 5J and Figure S10). Moreover, in *Tsc2*<sup>-/-</sup> cells, STAT3 was hyperactivated (Figure 5K). Inhibition of STAT3 via the selective inhib-

itor Stattic (Schust et al., 2006) reduced IL-10 production of LPS-treated monocytes but did not enhance production of TNF- $\alpha$  and IL-12p40 (Figure 5L). In agreement, Stattic failed to upregulate NF- $\kappa$ B activity in *Tsc2*<sup>+/+</sup> as well as *Tsc2*<sup>-/-</sup> cells (Figure 5M). Importantly, STAT3-deficient BMDMs (*Stat3*<sup>Lys-/-</sup>) showed diminished IL-10 production compared to control cells (*Stat3*<sup>fl/fl</sup>) after LPS stimulation, whereas rapamycin was still able to enhance IL-12p40 in these cells (Figure 5N). These results demonstrate that mTOR activates STAT3 to induce IL-10 production and independently inhibits NF- $\kappa$ B to restrain proinflammatory responses.

### mTOR Transcriptionally Controls Inflammatory Mediators and Negatively Regulates T Cell Responses

mTOR is considered to control primarily posttranscriptional events by modulating translation and mRNA stability (Hay and Sonenberg, 2004). However, the NF- $\kappa$ B hyperactivation described above (Figure 5) strongly indicated that mTOR regulates transcriptional processes in human monocytes. Accordingly, increased IL-12p40 and decreased IL-10 mRNA expression were observed in mTOR-inhibited human monocytes (Figures 6A and 6B). Rapamycin did not influence the stability of IL-12p40 or IL-10 mRNA (Figures 6C and 6D), substantiating the transcriptional control of IL-12p40 and IL-10 by mTOR.

To determine whether mTOR affects the transcription of other immunomodulatory molecules in myeloid cells, we performed microarray analysis of LPS-stimulated monocytes with or without rapamycin. After 4 hr of LPS stimulation, 290 genes were markedly modulated with rapamycin treatment (Table S1). Of these, 35 or 38 genes were more than 1.5-fold upregulated or downregulated, respectively. Analysis of transcription-factor binding sites revealed that genes containing NF- $\kappa$ B promoters were substantially overrepresented among the rapamycin-upregulated genes, corroborating with the finding that mTOR controls NF- $\kappa$ B activation on a genome-wide level (Figure S11).



**Figure 4. TSC2 Controls Pro- versus Anti-inflammatory Cytokine Production**

(A) Immunoblotting of whole-cell lysates from *Tsc2<sup>+/+</sup>* and *Tsc2<sup>-/-</sup>* MEFs was performed with the indicated antibodies.

(B) *Tsc2<sup>+/+</sup>* and *Tsc2<sup>-/-</sup>* cells were transfected with an IL-12p40 luciferase reporter plasmid and then stimulated with LPS as indicated. Relative luciferase activity per  $\mu$ g of protein is shown ( $n = 4$ ).

(C) *Tsc2<sup>+/+</sup>* and *Tsc2<sup>-/-</sup>* cells were stimulated with LPS for 5 hr and IL-10 mRNA was determined ( $n = 3$ ).

(D) *Tsc2<sup>+/+</sup>* and *Tsc2<sup>-/-</sup>* cells were stimulated with LPS for 24 hr, and production of IL-6 in the supernatants was determined ( $n = 3$ ).

(E) Immunoblot from THP-1 cells transfected with TSC2 or control siRNA and analyzed after 96 hr.

(F–H) THP-1 cells transfected with TSC2 or control siRNA for 72 hr were left unstimulated or were stimulated with LPS for 24 hr. Cell supernatants were analyzed for (F) IL-12p40, (G) TNF- $\alpha$ , and (H) IL-10 (mean  $\pm$  SEM;  $n = 3$ ). \* $p < 0.05$ .

To validate upregulation and downregulation of some of the differentially regulated genes, we assessed protein expression of genes that are important for the regulation of immune responses. Rapamycin prevented the upregulation of the negative T cell regulator PD-L1 (B7-H1) and the HIV coreceptor CCR5 (Figures 6E and 6F) but superinduced the costimulatory molecule CD86 on monocytes and peripheral myeloid DCs (Figure 6G and Figure S2B). CCL22, a T cell-attracting chemokine, was significantly upregulated by rapamycin, whereas the chemokine MCP-1, which is important for neoangiogenesis and induction of Th2 cell responses (Salcedo et al., 2000), was downregulated (Figures 6H and 6I). Conversely, in *Tsc2<sup>-/-</sup>* cells MCP-1 was upregulated (Figure S12). Thus, mTOR signaling controls the expression of a broad range of immunologically relevant genes in human monocytes.

The ability of mTOR to differentially regulate distinct cytokines, chemokines, and costimulatory molecules in myeloid cells suggest an impact on subsequent T cell activation. By employing an allogeneic T cell-activation model, we observed that rapamycin pretreatment of LPS-stimulated monocytes enhanced their T cell-stimulatory capacity (Figure 6J). Strikingly, analysis of T cell cytokine production revealed that IFN- $\gamma$  and IL-17 production was significantly higher in allogeneic T cells challenged with mTOR-inhibited monocytes (Figures 6K and 6L). Nevertheless, rapamycin suppressed T cell proliferation and IFN- $\gamma$  production when added directly to the mixed lymphocyte culture, reflecting its cell-cycle-blocking effect on T lymphocytes (data not shown). These results indicate that in myeloid immune cells mTOR negatively regulates the priming of Th1 and Th17 T cells.

**Inhibition of mTOR Protects Mice from a Lethal *Listeria monocytogenes* Infection**

We found that BMDMs from BALB/c and C57BL/6 mice, similarly to human cells, differentially regulated IL-12 and IL-10 production upon *L.m.* or LPS challenge under mTOR blockade in vitro (Figure 7A and Figure S13). To examine the physiological relevance of altered cytokine production after mTOR inhibition in vivo, we tested the effect of rapamycin on the immune response to *L.m.* in infected BALB/c mice. The increased susceptibility of BALB/c mice to *L.m.* results from an inherent inability to express sufficient amounts of IL-12, IFN- $\gamma$ , and IL-6 while producing excessive amounts of IL-10 (Dai et al., 1997; Seki et al.,

2002). Consequently, it has been shown that modifying the early cytokine imbalance in BALB/c mice is critical in controlling *L.m.* infection, even in mice lacking T and B cells (Tripp et al., 1994). Remarkably, administration of rapamycin for 3 days before receiving an LD<sub>100</sub> or LD<sub>50</sub> dose of *L.m.* significantly improved the survival of these mice compared with that of control mice given *L.m.* only (Figures 7B and 7C), even though the *L.m.*-specific T cell response was alleviated as expected (Figure S14). The T cell immunosuppressant FK506 did not modulate IL-12 or IL-10 production in vitro (Figure 3B) and also did not improve survival after *L.m.* infection in vivo (Figure 7B). Furthermore, depletion of macrophages and DCs with clodronate in vivo abolished the enhanced rapamycin-mediated resistance after *L.m.* infection (data not shown), indicating that myeloid immune cells are critical for the observed in vivo effects. Improved survival of rapamycin-treated mice infected with *L.m.* was confirmed by reduced bacterial burdens in liver and spleen 3 days after infection (Figure 7D). Interestingly, nitric oxide (NO), which is critical for clearing *L.m.* and whose production is under the control of NF- $\kappa$ B, was significantly increased in rapamycin-treated mice (Figure 7E). In addition, rapamycin-treated mice displayed reductions in the number and size of granulomatous lesions in the liver (Figure 7F). Hence, granulocyte infiltration was reduced to control numbers in the livers of rapamycin-treated animals (Figure 7G).

The *L.m.*-resistant phenotype in rapamycin-treated mice was accompanied by an increased production of IL-12p70 in blood early (4 hr) after infection (Figure 7H). At later time points (48 hr), the amount of serum IFN- $\gamma$  and IL-6 was also significantly greater in rapamycin-treated animals (Figure 7H). The expression of CD68, a murine monocyte and macrophage activation marker indicative of the production of large amounts of IL-12 (Mordue and Sibley, 2003), was examined. In rapamycin- and control-treated mice, comparable numbers of macrophages were present in the spleen 3 days after infection with *L.m.*, as shown by F4/80 staining (Figure 7I). However, a strong upregulation of CD68 on splenic macrophages was observed in rapamycin-treated animals (Figure 7I). Remarkably, rapamycin-treated infected mice demonstrated deficient IL-10 production by macrophages, whereas strong IL-10 expression was evident in control mice (Figure 7I). In accordance with these data, we found increased production of IL-12 and decreased production of IL-10 after in vitro restimulation with LPS of rapamycin-treated and *L.m.*-infected spleen cells (Figure S15). Collectively, these findings further demonstrate that mTOR inhibition in vivo can skew the pro- and anti-inflammatory cytokine response and protect susceptible BALB/c mice from a lethal *L.m.* bacterial infection.

## DISCUSSION

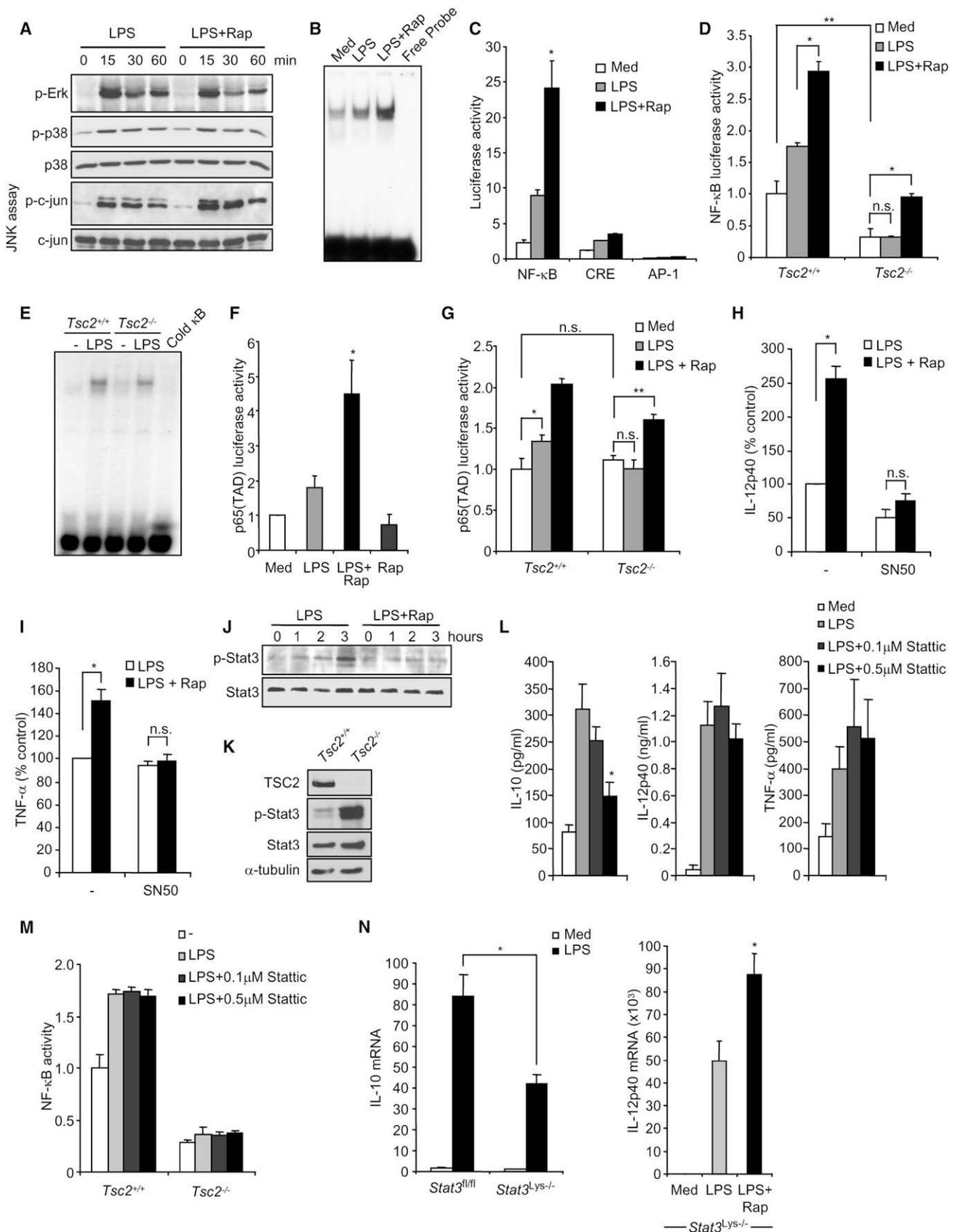
Although rapamycin was identified over 30 years ago, and inhibition of mTOR was approved as a therapeutic concept for the prevention of allograft rejection in 1999 (Miller, 1999), the physiological role of mTOR in mononuclear phagocytes remained largely unstudied. Our results have unraveled an unanticipated role of the mTOR signaling pathway in limiting proinflammatory cytokine production induced by various bacterial stimuli. Moreover, rapamycin treatment conveyed resistance to a lethal dose of

*L.m.* in susceptible mice demonstrating a direct benefit for the host when the cytokine balance is skewed by mTOR inhibition.

The present study shows that inhibition of mTOR promotes IL-12 and prevents IL-10 production. In this regard, activation of PI3K signaling was demonstrated recently to be an important negative regulatory pathway that dampens IL-12 and enhances IL-10 production after Toll-like receptor (TLR) stimulation (Fukao et al., 2002; Martin et al., 2003). Similarly, rapamycin promoted IL-23 production in human macrophages stimulated with *Mycobacterium tuberculosis* (Yang et al., 2006). Recently, NF- $\kappa$ B was found to be regulated by TSC2 as a survival factor to control cell survival (Ghosh et al., 2006). We identify the mTOR pathway as a pivotal negative regulator of NF- $\kappa$ B signaling but as a positive regulator of STAT3 signaling in myeloid innate immune cells. Activation of mTOR by inflammatory stimuli or by loss of TSC2 leads to decreased NF- $\kappa$ B but enhanced STAT3 activity, presumably providing a feedback loop to limit excessive inflammation. In this respect, it is interesting to note that the negative NF- $\kappa$ B regulator IKK- $\beta$  itself controls mTOR activity via TSC1 (Lee et al., 2007). How does the serine-threonine kinase mTOR control tyrosine phosphorylation of STAT3 and subsequently IL-10 production? Full activation of STAT3 occurs relatively late after 3 hr and depends on type I interferons (Chang et al., 2007; Ziegler-Heitbrock et al., 2003). Moreover, in murine macrophages, LPS-induced IL-10 production is completely dependent on the type I interferon receptor (IFNAR) (Chang et al., 2007). Because the mTOR pathway has been recently implicated to regulate type I interferon production (Colina et al., 2008), a sequential activation loop is conceivable, in which mTOR stimulates type I interferon, which subsequently activates STAT3 and IL-10 via IFNAR. These results provide a framework to study the complex regulatory networks that control the activation of NF- $\kappa$ B and STAT3 as well as the balance of IL-12 and IL-10 in innate phagocytic cells.

Currently, rapamycin is used as an alternative immunosuppressive treatment to calcineurin inhibitors to avoid or ameliorate chronic allograft damage (Halloran, 2004). However, distinct proinflammatory side effects have been recognized with the increased use of rapamycin, including lymphocytic alveolitis, interstitial pneumonitis, anemia associated with chronic inflammation, and severe forms of glomerulonephritis (Dittrich et al., 2004; Izzedine et al., 2005; Singer et al., 2000; Thauinat et al., 2005). The present findings may provide a basis for a better understanding of these peculiar inflammatory conditions that exist despite the potent immunosuppressive effects of mTOR inhibitors.

Recently, mTOR inhibitors have shown promising results in advanced clinical trials against certain malignancies including renal cell carcinoma, mantle cell lymphoma, and endometrial cancers (Faivre et al., 2006). Upon mTOR inhibition, we observed an altered cytokine milieu characterized by increased production of the antitumor cytokine IL-12 and IL-10 ablation, which is considered to foster escape from immunosurveillance. These results may add another dimension to explain the antitumor potency of mTOR inhibitors. Because the anticancer effects of mTOR inhibitors are attributed partially to their ability to prevent angiogenesis (Guba et al., 2002), it is interesting to note that rapamycin efficiently inhibited MCP-1, a chemokine that is produced by tumor-associated macrophages and that has been shown to promote neoangiogenesis (Salcedo et al., 2000).



In summary, we have identified and characterized a critical function of TSC2 and mTOR in regulation of the quality and quantity of the inflammatory reaction *in vitro* and *in vivo*. Activation of mTOR in mononuclear phagocytic cells enhances STAT3 activity and IL-10 but reduces proinflammatory molecules and NF- $\kappa$ B activation, whereas inhibition of mTOR with rapamycin has reciprocal effects. These findings suggest new therapeutic avenues for the regulation of pro- versus anti-inflammatory mediators, with potential relevance to cancer therapy, the design of novel adjuvants, and the control of distinct infectious and autoimmune diseases.

## EXPERIMENTAL PROCEDURES

### Reagents

LPS from *E. coli* 0111:B4, wortmannin, ATP, and UTP were from Sigma. SAC (PANSORBIN), SN50, Akt inhibitor I and IV, Stattic, and FK506 were from Calbiochem. Rapamycin was from Calbiochem, Sigma, LKT, or Wyeth Pharmaceuticals.

### Cell Culture

Human peripheral blood mononuclear cells (PBMCs) were isolated as described (Saemann et al., 2005). Monocytes were isolated from PBMCs by MACS with CD14 Microbeads (Miltenyi Biotec). Myeloid DCs were isolated with the BDCA-1 dendritic cell Isolation kit (Miltenyi Biotec). RPMI 1640 supplemented with 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin, and 10% FCS (Hyclone) was used as culture medium. We additionally added 50  $\mu$ M  $\beta$ -mercaptoethanol and 1 mM sodium pyruvate for THP-1 cells. THP-1 cells were pretreated with 1.2% DMSO for 24 hr before stimulation. Mouse embryonic fibroblasts (MEFs) were cultured in DMEM containing 4.5 g/L glucose, 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin, and 10% FCS. *Tsc2*<sup>+/+</sup> *p53*<sup>-/-</sup> and *Tsc2*<sup>-/-</sup> *p53*<sup>-/-</sup> MEFs were a kind gift of D. Kwiatkowski. *Stat3*<sup>fllox/fllox</sup> mice, backcrossed to C57BL/6, were cross-bred with B6.129P2-Lyz2<sup>tm1(Cre)fl/J</sup> (The Jackson Laboratory) for generating *Stat3*<sup>lys-/-</sup> and *Stat3*<sup>fl/fl</sup> (littermate controls) mice. BALB/c and C57BL/6 mice were purchased from Harlan Winkelmann. BMDMs from were isolated and grown as described (Strobl et al., 2005) and were washed 1 day before stimulation.

### Measurement of Cytokine Production

We pretreated  $1 \times 10^6$  cells for 90 min with the indicated concentrations of rapamycin, wortmannin, stattic, 10nM FK506, or 18  $\mu$ M SN50 and then stimulated them with 100 ng/ml LPS ( $\pm$ 30 ng/ml IFN- $\gamma$  as indicated), 75  $\mu$ g/ml SAC, or  $10^7$  *L.m.* in 24-well plates. Cell-free supernatants were collected after 20–24 hr. In some experiments, human monocytes were cultured in macrophage-SFM medium (Invitrogen), incubated with 1% amino acids, 10  $\mu$ M ATP, or 10  $\mu$ M UTP overnight, and stimulated with 20  $\mu$ g/ml SAC. Cytokines

were measured by ELISA to IL-12p40, IL-12p70, IL-10, TNF- $\alpha$ , IL-6, IL-17, IFN- $\gamma$  (all R&D Systems), and IL-23 as well as MCP-1 (Bender MedSystems). Murine cytokines were determined by the Luminex bead system with beads from Upstate and read on a Luminex 100 reader.

### Analysis of Signal Transduction Events

A total of  $1 \times 10^7$  monocytes,  $1 \times 10^7$  BMDM, or 70% confluent MEFs starved overnight were treated and stimulated as indicated. Extract preparation and western blotting was done as described (Saemann et al., 2005). Antibodies were p-mTOR (Ser2448), p-p70S6K (Thr389), p70S6K, p-4E-BP1 (Thr37/46), 4E-BP1, p-TSC2 (Ser939), Raptor, p-p38 (Thr180/Tyr182), p-S6 (Ser 240/244), p-STAT3 (Tyr705), STAT3 (all Cell Signaling Technology), p-Erk (Tyr204), and p38 (Santa Cruz Biotechnology). For detection of p-mTOR by FACS, monocytes were fixed with 2% formaldehyde, permeabilized with 0.1% saponin, and stained with p-mTOR and Alexa-Fluor-448-labeled goat anti-rabbit IgG (Molecular Probes). JNK activity was determined with the SAPK/JNK Kinase Assay Kit (Cell Signal Technology).

### Immunofluorescence Microscopy

Cells were applied to poly-L-lysine-coated slides (Marienfeld), fixed with 4% paraformaldehyde, quenched with 100 mM glycine cells, permeabilized with methanol, blocked with 1% BSA, and stained with p-S6 antibody or isotype control overnight at 4°C. Cells were stained with Alexa-Fluor-448-labeled goat anti-rabbit IgG (Invitrogen), underwent nuclear tracking with 0.5  $\mu$ g/ml Hoechst-33342 (Invitrogen), and were mounted in Vectashield mounting medium.

### RNAi

THP-1 cells were transfected in full medium without antibiotics with 100 nM ON-TARGETplus SMARTpool human TSC2 or 100 nM ON-TARGETplus siCONTROL nontargeting pool (Dharmacon) with Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's protocol. Cells were used after 72 hr.

### Electrophoretic Mobility-Shift Assay

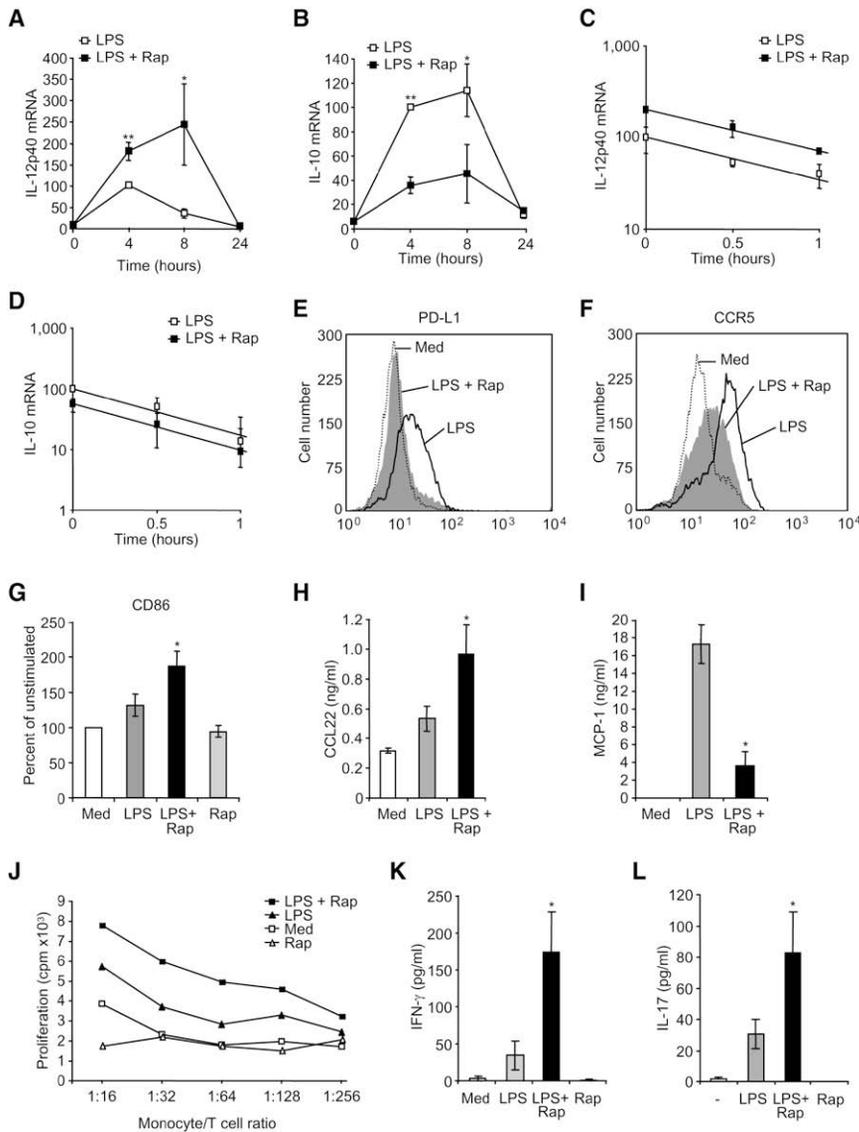
Electrophoretic mobility-shift assay was performed as described (Saemann et al., 2005). Oligonucleotides resembling the consensus binding site for NF- $\kappa$ B, STAT3, and AP-1 as well as antibodies for supershift assays were from Santa Cruz Biotechnology.

### Reporter-Gene Assays

Reporter constructs for NF- $\kappa$ B, AP-1, and CRE were from Stratagene. p65(TAD) and Gal4-Luc plasmids were a kind gift of N. Perkins. A renilla luciferase construct was cotransfected for expression normalization. THP-1 cells were transfected with Lipofectamine LTX and Plus Reagent for 24 hr. MEFs were transfected with Lipofectamine for 3–5 hr. Cells were treated and stimulated for 20 hr. Lysates were prepared with passive lysis buffer (Promega) and assayed for luciferase activity.

## Figure 5. The mTOR Pathway Regulates NF- $\kappa$ B and STAT3

- (A) Human monocytes were preincubated with rapamycin or medium and then stimulated with LPS for the indicated time points. Cell lysates were analyzed by immunoblotting or for JNK activity.
- (B) DNA-binding activity of NF- $\kappa$ B in nuclear extracts from monocytes, which were stimulated for 4 hr, was analyzed by EMSA.
- (C) THP-1 cells were transfected with NF- $\kappa$ B, CRE, or AP-1 luciferase reporter plasmids and treated as indicated. Luciferase activity is shown as the mean  $\pm$  SEM of three independent experiments performed in triplicate.
- (D) *Tsc2*<sup>+/+</sup> and *Tsc2*<sup>-/-</sup> cells were transfected with an NF- $\kappa$ B reporter plasmid and stimulated as indicated. Luciferase activity is shown as the mean  $\pm$  SEM of three independent experiments performed in triplicate.
- (E) NF- $\kappa$ B DNA-binding activity in nuclear extracts from *Tsc2*<sup>+/+</sup> and *Tsc2*<sup>-/-</sup> cells, which were stimulated for 2 hr with LPS, was analyzed by EMSA.
- (F and G) THP-1 (F) or *Tsc2*<sup>+/+</sup> and *Tsc2*<sup>-/-</sup> (G) cells were transfected with a Gal4 luciferase reporter and an expression plasmid encoding a p65(TAD)-Gal4 fusion. Luciferase activity is shown as the mean  $\pm$  SEM of three independent experiments performed in triplicate.
- (H and I) Human monocytes were treated with medium, the NF- $\kappa$ B inhibitor SN50, or rapamycin as indicated and stimulated with LPS. The concentration of IL-12p40 (H) and TNF- $\alpha$  (I) in the supernatant was determined by ELISA. Bars represent percentage  $\pm$  SEM of the LPS-stimulated control performed in duplicate (n = 3). \*p < 0.05, \*\*p < 0.01 compared with the respective LPS controls, or as indicated. n.s., not significant.
- (J) Human monocytes were treated as indicated. Cell lysates were analyzed for STAT3 phosphorylation.
- (K) Immunoblotting of cell lysates from *Tsc2*<sup>+/+</sup> and *Tsc2*<sup>-/-</sup> MEFs was performed with the indicated antibodies.
- (L) Monocytes were treated with the indicated amounts of Stattic and then stimulated with LPS (n = 3).
- (M) *Tsc2*<sup>+/+</sup> and *Tsc2*<sup>-/-</sup> cells were transfected with an NF- $\kappa$ B reporter plasmid and stimulated as indicated (n = 2).
- (N) *Stat3*<sup>lys-/-</sup> and *Stat3*<sup>fl/fl</sup> BMDM were stimulated with rapamycin and/or LPS as indicated for 4 hr. IL-10 and IL-12p40 mRNA levels were assessed by RT-PCR (n = 3).



**Figure 6. Modulation of Inflammatory Mediators at the Transcriptional Level by Rapamycin in Human Monocytes and the Impact on T Cell Activation**

(A and B) Kinetics of (A) IL-12p40 and (B) IL-10 mRNA in monocytes were assessed by RT-PCR. mRNA levels are depicted as percentage of the 4 hr LPS-stimulated samples (mean  $\pm$  SEM; n = 4). (C and D) Monocytes were pretreated with rapamycin or medium and then stimulated with LPS for 2 hr. Subsequently, actinomycin D was added, and degradation of (C) IL-12p40 and (D) IL-10 was monitored by real-time PCR. Results depict one representative example  $\pm$  SEM of four donors.

(E–G) Monocytes were pretreated with rapamycin and stimulated with LPS for 48 hr. PD-L1 (E), CCR5 (F), and CD86 (G) surface expression was analyzed by flow cytometry. Results are representative of four independent (E and F) or represent the mean of five separate experiments (G).

(H and I) Monocytes were treated as indicated. Production of (H) CCL22 and (I) MCP-1 was determined by ELISA (n = 4).

(J–L) Monocytes were pretreated with rapamycin or medium and stimulated with LPS for 24 hr. Cells were cocultured with allogeneic T cells at the indicated ratios.

(J) Proliferation was measured on day 6 and is expressed as the mean of triplicate cultures. One representative experiment of four is shown.

(K and L) Monocytes were treated as described, washed, and added to allogeneic T cells at a ratio of 1:4. IFN- $\gamma$  and IL-17 in the cultures was determined on day 2 and 6, respectively (mean  $\pm$  SEM; n = 3). \*p < 0.05, \*\*p < 0.01 compared with the respective LPS controls.

**Listeria monocytogenes Infection Model**

Female BALB/c mice (7–8 weeks) were injected i.p. with 1.5 mg/kg/day rapamycin. Rapamycin was suspended at 1 mg/ml in sterilized medium (1 g sodium carboxymethylcellulose (Sigma-Aldrich) and 1.25 g Tween 80 (Sigma) per 500 ml deionized water) with an ultrasound bath. The stock solution was diluted to 300  $\mu$ g/ml with PBS for injection. *L.m.* serovar 1/2 A was cultured in brain-heart infusion broth. For the survival model, daily treatment of BALB/c mice with rapamycin or medium alone started 3 days before logarithmically dividing *L.m.* were injected i.p. and continued until the end of the experiment. Rapamycin did not affect growth of *L.m.* (data not shown). All studies were approved by the official Austrian ethics committee for animal experiments (GZ.68.205/67-BRGT/2003; GZ.68.205/171-BRGT/2005).

**Determination of Bacterial Load, Nitric Oxide Production, and Granulocyte Infiltration**

We determined viable bacteria in liver homogenates by plating serial dilutions on Oxford Listeria selective agar plates (Merck). Serum nitrate and nitrite levels were determined with the Griess reagent (Strobl et al., 2005). Myeloperoxidase activity in liver homogenates was monitored at 560 nm.

**Histology and Immunohistochemistry**

Liver and spleen of mice were removed on day 3, fixed in 4.5% phosphate-buffered formaldehyde, and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E) (100 $\times$  magnification). For immunohistochemical detection of proteins (200 $\times$  magnification), rat anti-mouse

**Flow Cytometry**

Cells were incubated with antibodies (see figure legends) and analyzed on a FACSCalibur. Anti-PD-L1 was kindly provided by O. Majdic.

**T Cell Stimulation**

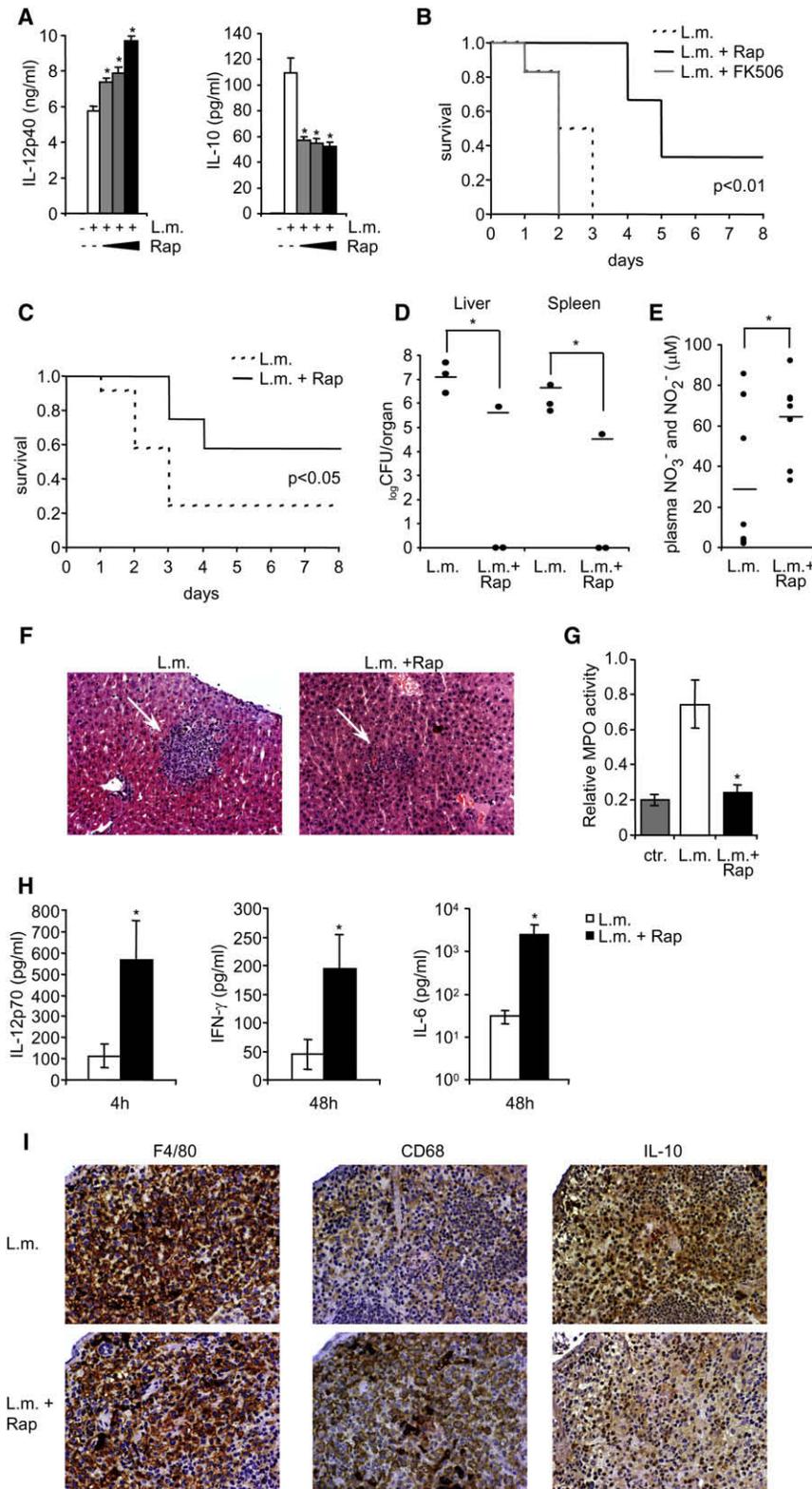
Allogeneic T cell activation was performed as described (Saemann et al., 2005).

**Quantitative RT-PCR**

RNA from  $2 \times 10^6$  monocytes or 70% confluent MEFs was extracted in TRIzol (Invitrogen). cDNA was generated by Superscript II (Invitrogen). mRNA levels were determined by TaqMan Gene Expression Assays (Applied Biosystems) on a ABI Prism 7000 and normalized to 18S rRNA. For measurement of mRNA stability, 10  $\mu$ g/ml actinomycin D was added to the medium- or rapamycin-pretreated monocytes 3 hr after stimulation with LPS and RNA was extracted at the indicated time points.

**Microarrays and Data Analysis**

A total of  $10^7$  CD14<sup>+</sup> monocytes from four different donors were stimulated for 4 hr. Cells were frozen at  $-80^\circ\text{C}$ . Sample preparation and hybridization to a PIQOR Immunology Microarray Human Antisense was performed at the Miltenyi Microarray Service Unit.



**Figure 7. Rapamycin Protects Mice from *Listeria monocytogenes* Infection and Modulates Innate Immunity**

(A) BMDMs from BALB/c mice were treated with 10, 100, or 500 nM rapamycin (Rap) and *L.m.* as indicated. IL-12p40 and IL-10 in the supernatants were determined by ELISA.

(B and C) BALB/c mice were pretreated i.p. with rapamycin, FK506, or medium for 3 days as indicated and then challenged with (B)  $10^7$  *L.m.* (n = 6) or (C)  $10^6$  *L.m.* (n = 12). Survival was monitored for 8 days. Statistics are indicated as p values in the figures. One representative experiment of two independent experiments is shown.

(D) BALB/c mice were pretreated as above and challenged with  $10^5$  *L.m.* On day 3, the numbers of bacteria in the spleen and liver were determined (n = 3).

(E–H) BALB/c mice were pretreated as above and challenged with  $10^4$  *L.m.* As shown in (E), we assessed plasma levels of nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) as a marker of nitric oxide production (n = 8–9). As shown in (F), on day 3, liver histomorphology was analyzed by H&E staining. Granulomatous lesions are indicated (by arrows). (G) shows relative myeloperoxidase (MPO) activity in the livers of control and infected mice with or without rapamycin. As shown in (H), serum concentrations of IL-12p70, IFN- $\gamma$ , and IL-6 were determined from infected animals 4 and 48 hr postinfection (mean  $\pm$  SEM; n = 3).

(I) Spleens were analyzed by immunohistochemical staining of F4/80, CD68, and IL-10 on day 3 postinfection. \*p < 0.05 compared with the respective *L.m.* controls.

**Statistics**

Cytokine levels were compared with Student's t test. Survival data shown as Kaplan Meier plots were analyzed with the log-rank test for the comparison of the groups.

**ACCESSION NUMBERS**

The complete data set has been submitted to the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>; submission #GSE6002).

**SUPPLEMENTAL DATA**

Supplemental Data include fifteen figures and one table and can be found with this article online at <http://www.immunity.com/cgi/content/full/29/4/565/DC1/>.

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F4/80 and CD68 (Serotec) and goat anti-rat IL-10 (R&D Systems) were used. A biotin-streptavidin-horseradish peroxidase-based method was used for detection of the primary antibodies.

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