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Toxoplasma gondii Interferes with Lipopolysaccharide-Induced Mitogen-Activated Protein Kinase Activation by Mechanisms Distinct from Endotoxin Tolerance[1]

Leesun Kim, Barbara A. Butcher, and Eric Y. Denkers[2]

We show in this study that *Toxoplasma gondii* infection induces rapid activation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2, and stress-activated protein kinase/c-Jun N-terminal kinase MAPK, followed promptly by their deactivation in mouse macrophages. Nevertheless, when infected cells were subsequently subjected to LPS triggering, MAPK activation was severely defective, in particular in the case of p38 MAPK, which is required for LPS-triggered TNF-α and IL-12 production. Similar effects occurred during endotoxin tolerance, but the phenomena were distinct. LPS pretriggering failed to activate the major p38 MAPK kinase, MAPK kinase 3/6. *Toxoplasma* infection, in contrast, resulted in sustained activation of this kinase. Furthermore, endotoxin pre-exposure blocked IκBα degradation upon subsequent LPS triggering, but this was not the case for *Toxoplasma* preinfection. Endotoxin-mediated down-regulation of the LPS receptor, Toll-like receptor 4, has been suggested as one possible mechanism contributing to tolerance, and we found in this study that LPS down-modulated Toll-like receptor 4 expression. In contrast, *Toxoplasma* infection induced up-regulation of this pattern recognition receptor. Our results show that *T. gondii* blocks LPS-triggered cytokine production in part through MAPK inactivation, and that this occurs through pathways distinct from endotoxin-induced tolerance.   *The Journal of Immunology,* 2004, 172: 3003–3010.

The intracellular protozoan *Toxoplasma gondii* is an opportunistic pathogen that occurs throughout the world in human and animal populations. Infection proceeds through an acute phase during which proliferating tachyzoites disseminate through host tissues, to a chronic stage, in which long-lived cysts harbor quiescent bradyzoites, predominantly in tissues of the skeletal muscle and CNS (1). *Toxoplasma* elicits strong protective immunity characterized by high level IFN-γ production (2–4). This response is necessary to provide resistance to infection, but must be tightly regulated to avoid the harmful consequences of proinflammatory cytokine overproduction (5–8).

Toxoplasma manipulates host signaling cascades during intracellular infection, and in macrophages (Mϕ)[3] and dendritic cells it is likely that this represents a general strategy to control proinflammatory responses (9). For example, the parasite down-modulates signaling pathways leading to programmed cell death (10, 11) and can block the function of STAT-1, resulting in impaired MHC Ag presentation to T cells (12, 13). In addition, *T. gondii* blocks both IL-12 and TNF-α production, and this may relate to the ability of the parasite to prevent NF-κB nuclear translocation

(14, 15). Insofar as TNF-α production remains suppressed despite the fact that NF-κB translocation is eventually restored, it can be inferred that additional signaling pathways must be disabled by *T. gondii*.

Mitogen-activated protein kinase (MAPK) signal transduction pathways are highly conserved cascades important in diverse aspects of the immune response (16). The MAPK form a family of protein kinases that include extracellular signal-regulated kinase (ERK), p38 MAPK and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). MAPK are activated by phosphorylation of Thr-X-Tyr tripeptide motifs mediated by upstream MAPK kinases (MKK). Phosphorylated MAPK, in turn, activate several transcription factors, including NF-IL-6, Elk-1, c-Jun, and activating transcription factor 2 (ATF-2) or other protein kinases, such as MAPK-activated protein kinase 2 (17). Activation of MAPK is tightly regulated through the induction of dual-specificity MAPK phosphatases that may themselves be induced by ERK1/2 and p38 MAPK pathways (18–20). Gram-negative LPS is a potent Mϕ stimulus of the three major MAPK. Studies with small molecule inhibitors of p38 MAPK as well as MKK3$^{-/-}$ mice that lack a major upstream kinase of p38 MAPK have implicated the p38 MAPK signaling cascade in Mϕ TNF-α and IL-12 production during LPS stimulation (21–24).

Mϕ that undergo triggering by LPS and other Toll-like receptor (TLR) ligands display down-modulated MAPK activation and cytokine production subsequent to the initial response, a phenomenon termed endotoxin tolerance (25). Induction of MAPK phosphatases, interference with biochemical signaling immediately downstream of TLR, induction of NF-κB p50 homodimers, and down-regulation of TLR4 are mechanisms that have been implicated in the tolerance phenomenon (19, 25–29). In an in vivo situation, endotoxin tolerance may be required to prevent persistent TLR ligand-dependent Mϕ activation that would otherwise be brought about by continual exposure to commensal flora or TLR-expressing microorganisms that establish chronic infections.

In this study we show that *Toxoplasma* infection results in activation of p38 MAPK, ERK1/2, and SAPK/JNK MAPK. Nevertheless, when infected cells are subjected to LPS triggering, reactivation of MAPK is severely impaired. The effect is similar to that occurring during endotoxin tolerance, but the underlying mechanisms are distinct. Therefore, interference with MAPK signaling is an additional way that *T. gondii* prevents proinflammatory cytokine production during intracellular infection.

Materials and Methods

Mice

C57BL/6 female mice, 6–8 wk age, were purchased from Taconic Farms (Germantown, NY). The mice were housed under specific pathogen-free conditions in the Cornell University College of Veterinary Medicine animal facility, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Parasites

RH and ts-4 strain tachyzoites were maintained by biweekly passage on human foreskin fibroblast monolayers in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 1% heat-inactivated FCS (HyClone, Logan, UT), 100 U/ml penicillin (Life Technologies), and 0.1 mg/ml streptomycin (Life Technologies). Transgenic RH parasites expressing yellow fluorescent protein (YFP) were provided by Dr. D. Roos (University of Pennsylvania, Philadelphia, PA).

Bone marrow-derived Mϕ preparation

Bone marrow cells were flushed from femur and tibia and cultured in medium consisting of DMEM, 10% heat-inactivated FCS, 5% horse serum (Life Technologies), 2 mM glutamine, 1 mM sodium pyruvate (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in the presence of 30% supernatant from L929 cells as a source of M-CSF. After 4 days of culture, nonadherent cells were removed, and adherent monolayers were washed in PBS, resuspended by gentle scraping in ice-cold PBS, then replated in DMEM with 1% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Cell culture

Infection of day 5 Mϕ was accomplished by addition of RH parasites (6:1 ratio of parasites to cells) to cell cultures. Plates were briefly centrifuged (3 min, 200 × *g*) to synchronize contact between tachyzoites and Mϕ. After 6-h incubation (37°C), LPS (*Escherichia coli* strain 055:B5; Sigma-Aldrich, St. Louis, MO) was added (100 ng/ml), and at varying times cells were collected for biochemical assays. For measurement of cytokine release, LPS was removed after 30 min, cells were gently washed, and fresh medium was added. In some experiments parasite infection and LPS addition were performed without subsequent stimulation. In other experiments, cells were subjected to LPS pretriggering (100 ng/ml), then 6 h later cells were re-exposed to LPS (100 ng/ml). MAPK inhibition studies were accomplished by addition of SB202190 (Calbiochem, San Diego, CA) or the equivalent solvent concentration to cells 2 h before stimulation (LPS; 25 ng/ml). After 30-min stimulation, LPS was removed, fresh medium, including MAPK inhibitor, was added, and supernatants were collected 5.5 h later for cytokine ELISA.

Immunoblotting

Cells (2 × 10^6/sample) were lysed in reducing SDS sample buffer, and DNA was sheared by forcing samples three times through a 27-gauge needle, followed by centrifugation (18,000 × *g*, 5 min). After boiling for 3 min, cellular lysates were separated by 10% SDS-PAGE, and proteins were subsequently electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were then blocked in 5% nonfat dry milk (Nestle USA, Solon, OH) containing 0.1% Tween 20 (Sigma-Aldrich) in Tris-buffered saline, pH 7.6 (TBST), for 1 h at room temperature, followed by incubation with Ab specific for dual-phosphorylated (active) and total ERK1/2, p38 MAPK, and SAPK/JNK or IκBα (used at the concentrations recommended by the manufacturer; Cell Signaling Technology, Beverly, MA) in 5% BSA in TBST overnight at 4°C. After washing blots in TBST, Ab were detected with an HRP-conjugated secondary Ab (Cell Signaling Technology) in TBST containing 5% nonfat dry milk for 1 h at room temperature. After washing in TBST, bands were visualized using an ECL system (Lumi-GLO; Cell Signaling Technology). Detection of mitogen-activated protein kinase phosphatase-1 (MKP-1) was similarly accomplished following the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro MAPK assay

MAPK activity was measured by in vitro kinase assay employing commercially available kits and following the manufacturer's instructions (Cell Signaling Technology). Briefly, for ERK1/2 and p38 MAPK, phosphorylated MAPK were immunoprecipitated with phospho-specific Ab-conjugated agarose beads. For the SAPK/JNK assay, c-Jun fusion protein bound to Sepharose beads allowed precipitation of phosphorylated SAPK/JNK. After washing in lysis buffer, precipitates were resuspended in 50 μl of kinase buffer with 100 μM (SAPK/JNK) or 200 μM (ERK1/2 and p38 MAPK) ATP. For p38 and ERK1/2 MAPK assays, 2 μg of recombinant substrates (ATF-2 and Elk-1, respectively) were included in the reaction. After 30-min incubation at 30°C, reducing SDS sample buffer was added, and samples were subjected to Western blot analysis using phospho-specific anti-c-Jun, ATF-2, and Elk-1 Ab as described above.

Immunofluorescence

Bone marrow-derived Mϕ were seeded onto sterile 12-mm coverslips in 24-well plates. After 6-h pretreatment with medium, LPS (100 ng/ml), or tachyzoite (6:1 ratio of parasites to cells), cells received secondary LPS stimulation (100 ng/ml) for 60 min. Mϕ were fixed with 3% formaldehyde and blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 0.075% saponin (Sigma-Aldrich). Then cells were incubated with goat anti-p65 Ab (Santa Cruz Biotechnology) and mouse anti-p30 (SAG-1) Ab (Argene, North Massapequa, NY) for 30 min at room temperature, followed by FITC-conjugated donkey anti-goat Ig and Texas Red-conjugated goat anti-mouse Ig for another 30 min (both secondary Ab from Molecular Probes, Eugene, OR). DAPI was added for the last 10 min to stain nuclei. All images were collected with a BX51 fluorescence microscope (Olympus, Melville, NY) and DP70 camera at ×80 magnification using DP controller software.

Flow cytometry

To examine Mϕ TLR4 surface expression, cells were stimulated with LPS or were infected with YFP-expressing tachyzoites. Mϕ were collected and, after blocking with 10% normal donkey serum (Jackson ImmunoResearch Laboratories) in PBS, were incubated with rat anti-mouse TLR4/MD2 Ab (1 μg/ml; Santa Cruz Biotechnology) for 30 min on ice. This was followed by incubation with secondary PE-conjugated donkey anti-rat Ig (Jackson ImmunoResearch Laboratories) for 30 min on ice. The cells were analyzed on a FACSCalibur flow cytometer, and CellQuest software (BD Immunocytometry Systems, San Jose, CA) was used for data analysis.

Cytokine ELISA

IL-12 p40 was measured by ELISA as described previously (14), and TNF-α was measured using a commercial kit according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Results

T. gondii *infection and p38 MAPK inhibition block LPS-induced TNF-α and IL-12(p40) production*

We previously demonstrated a strong down-regulatory effect of *T. gondii* preinfection on subsequent secondary LPS-triggered TNF-α and IL-12 production. Down-modulation was apparent when LPS triggering was performed after 2 h (14) as well as 6 h (Fig. 1A) of infection. In this study we compared the down-regulatory effects of 6-h *Toxoplasma* infection with 6-h LPS prestimulation. As shown in Fig. 1*B*, when Mϕ were subjected to 6-h LPS pretriggering, followed by addition of fresh LPS-containing medium, there was no additional production of TNF-α in supernatants collected 6 h after secondary stimulation. For IL-12, cells continued to release the cytokine after LPS primary stimulation even when the secondary incubation was in medium alone. In addition, secondary LPS stimulation failed to increase the amount of IL-12 released, again suggesting a failure to respond to secondary stimulation (Fig. 1*B*).

To begin addressing the molecular basis for these effects, we examined the involvement of p38 MAPK that is implicated in Mϕ proinflammatory cytokine production (21–24). Addition of
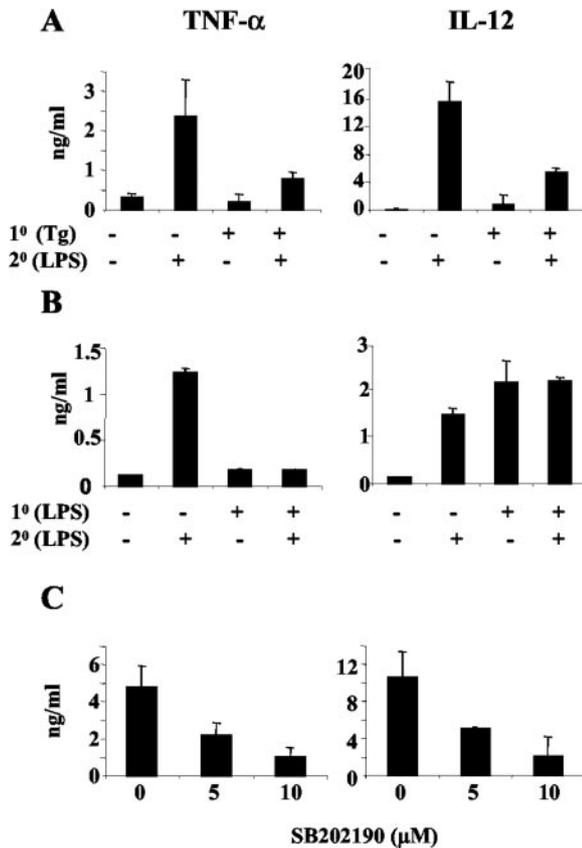
FIGURE 1. *T. gondii* infection and p38 MAPK inhibition block LPS-induced TNF-α and IL-12(p40) production. *A*, Bone marrow-derived Mφ were given a primary infection with RH strain tachyzoites (Tg; 1:6 ratio of cells to parasites) or were cultured in medium alone. After 6 h, cells received a secondary LPS stimulation (100 ng/ml) or fresh medium. *B*, Mφ prestimulated with LPS (100 ng/ml) or medium, followed 6 h later by addition of fresh LPS or medium as secondary stimulation. *A* and *B*, Supernatants were collected for cytokine ELISA 6 h after secondary stimulation. *C*, Bone marrow-derived Mφ were pretreated for 2 h with the p38 MAPK inhibitor, SB202190, followed by LPS stimulation (25 ng/ml) for 30 min. After 5.5 h, IL-12(p40) and TNF-α release was measured by ELISA. These experiments were repeated twice with the same result.

SB202190, a highly potent and specific inhibitor of p38 MAPK (30, 31), to Mφ triggered with LPS in the absence of *T. gondii* resulted in dose-dependent suppression of both TNF-α and IL-12(p40) (Fig. 1*C*). We conclude that *Toxoplasma* disables LPS-triggered signaling pathways leading to TNF-α and IL-12 in bone marrow-derived Mφ, and that these transduction pathways themselves involve p38 MAPK.

*LPS and* T. gondii *induce rapid Mφ MAPK phosphorylation*

We next asked whether *Toxoplasma* itself activates Mφ MAPK activity. As shown in Fig. 2*A*, phosphorylation of p38 MAPK, ERK1/2, and SAPK/JNK occurred within 10–20 min of both LPS stimulation and tachyzoite infection, and this was followed by dephosphorylation of the MAPK. For LPS stimulation, it is interesting to note that p38 MAPK and SAPK/JNK dephosphorylation was more complete than that of ERK1/2. Induction of MKP-1, a phosphatase with the specificity p38 MAPK = SAPK/JNK ≪ ERK1/2, has been implicated in this pattern of dephosphorylation during endotoxin stimulation (20, 32). Indeed, we found that LPS triggered appearance of MKP-1 at 60 min poststimulation, correlating with the disappearance of active p38 MAPK and SAPK/JNK (Fig. 2*B*). *T. gondii* induced a distinct pattern of phosphorylation. Each MAPK was activated within 10 min of infection, p38 MAPK as well as ERK1/2 underwent dephosphorylation within 30 min, and SAPK/JNK dephosphorylation occurred with slower kinetics (Fig. 2*A*). In contrast to LPS, we found no evidence for induction of MKP-1 after *Toxoplasma* infection (Fig. 2*B*).

LPS or T. gondii *pre-exposure blocks subsequent LPS induced rephosphorylation of MAPK*

We determined whether *Toxoplasma*-infected cells could undergo MAPK rephosphorylation in response to secondary LPS challenge. In these experiments, Mφ were triggered with endotoxin 6 h after either infection or LPS stimulation. This time point was chosen to assure that the MAPK had undergone deactivation after initial phosphorylation. In addition, as shown in Fig. 1*A*, cells infected for 6 h are suppressed in their ability to make TNF-α and IL-12 upon subsequent LPS triggering.

Both *Toxoplasma* and endotoxin pre-exposure induced MAPK nonresponsiveness to subsequent LPS stimulation, as shown by immunoblot analysis with phospho-specific anti-MAPK Ab. The

FIGURE 2. LPS and *T. gondii* induce rapid Mφ MAPK phosphorylation. Bone marrow-derived Mφ were either stimulated with LPS (100 ng/ml) or infected with tachyzoites (1:6 ratio of cells to parasites), then at the indicated time points whole cell lysates were collected in SDS sample buffer. Lysates were subjected to SDS-PAGE, followed by Western blotting using Ab specific for phosphorylated MAPK and phosphorylation-state independent anti-MAPK Ab (*A*) or anti-MKP-1 Ab (*B*). *B*, The *upper band* corresponds to MKP-1. The *lower band* represents a nonspecific Ab-reactive protein and is included as a control for equivalent sample loading. The results are representative of three independent experiments.
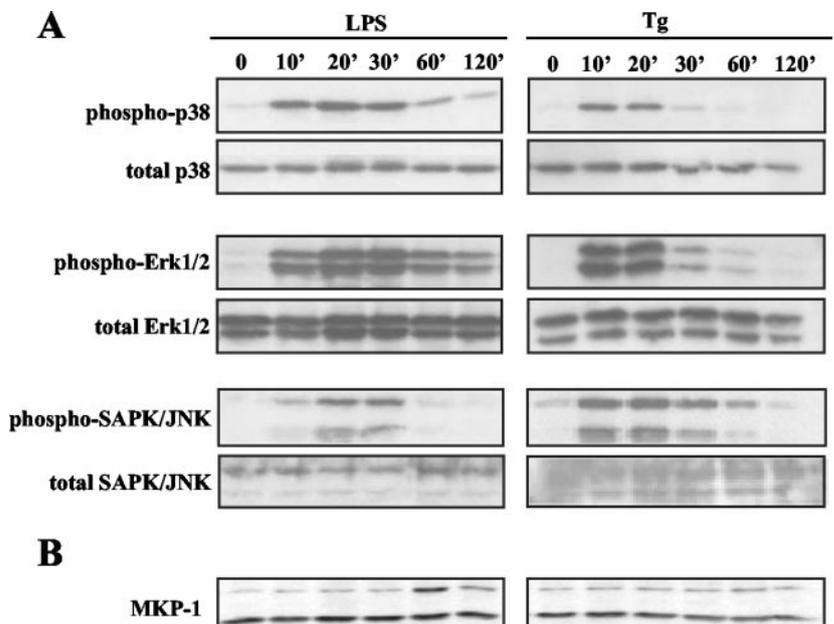
effects of preinfection were particularly striking for p38 MAPK (Fig. 3*A*). In this study *T. gondii* almost completely blocked rephosphorylation of this molecule. LPS pre-exposure also resulted in defective p38 MAPK reactivation, although the effects were less striking. In contrast, although LPS prestimulation resulted in severely impaired ERK1/2 rephosphorylation, the effects of *T. gondii* preinfection were less profound. Parasite infection also had a dramatic effect on the ability of SAPK/JNK to undergo LPS-induced reactivation (Fig. 3*A*).

We confirmed that 6-h *T. gondii* preinfection resulted in decreased MAPK activity by performing in vitro kinase assays on immunoprecipitated phospho-p38 MAPK, phospho-ERK1/2, and phospho-SAPK/JNK. As shown in Fig. 3*B*, ATF-2 (p38 substrate), Elk-1 (ERK1/2 substrate), and c-Jun (SAPK/JNK substrate) were strongly phosphorylated by MAPK precipitated from lysates of 30 min LPS-stimulated cells. Consistent with the data shown in Fig. 3*A*, by 6 h after *T. gondii* infection there was little or no detectable kinase activity for any of the three MAPK. In addition, the amount of active MAPK present in lysates from infected cells that were subsequently stimulated with LPS was substantially less.
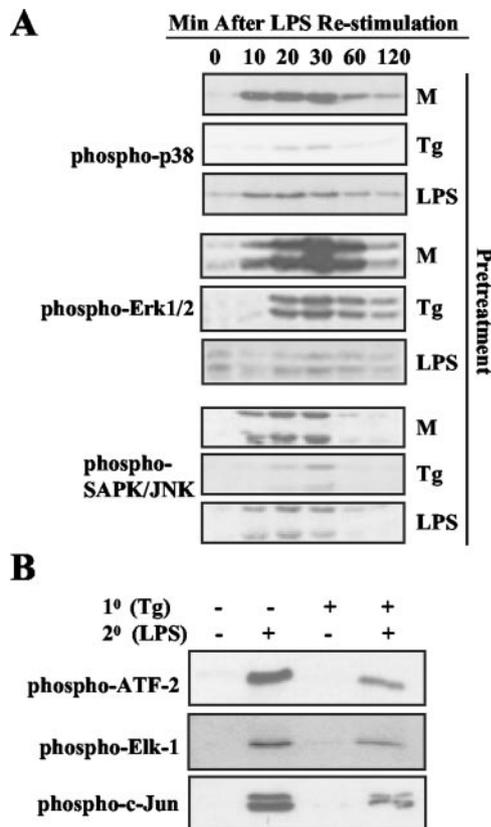
FIGURE 3. LPS or *T. gondii* pre-exposure blocks subsequent LPS induced rephosphorylation of MAPK. *A*, For pretreatment, Mϕ were left untreated (M), infected with *T. gondii* (Tg; 1:6 ratio of cells to tachyzoites), or stimulated with LPS (100 ng/ml), then after 6 h cells were restimulated with LPS (100 ng/ml) for the indicated time intervals. MAPK phosphorylation was determined by immunoblot analysis. *B*, Cells were subjected to primary *T. gondii* infection or were left in medium alone. Six hours later Mϕ were subjected to secondary 30-min stimulation with LPS or medium alone. MAPK activity was measured by immunoprecipitating phosphorylated MAPK and examining the ability to phosphorylate exogenous substrates using phospho-specific Ab recognizing ATF-2, Elk-1, and c-Jun. The experiment was repeated three times with similar results.

LPS and T. gondii *trigger distinct MKK3/6 activation kinetics*

Because of the importance of p38 MAPK in LPS-induced cytokine production (Fig. 1*C*), we sought to determine whether the faulty reactivation of this kinase could be attributed to defective activation of MKK3/6, which is a major upstream kinase of p38 MAPK. As shown in Fig. 4*A*, LPS triggered phosphorylation of MKK3/6 at ∼20 min poststimulation, and this was followed by a gradual
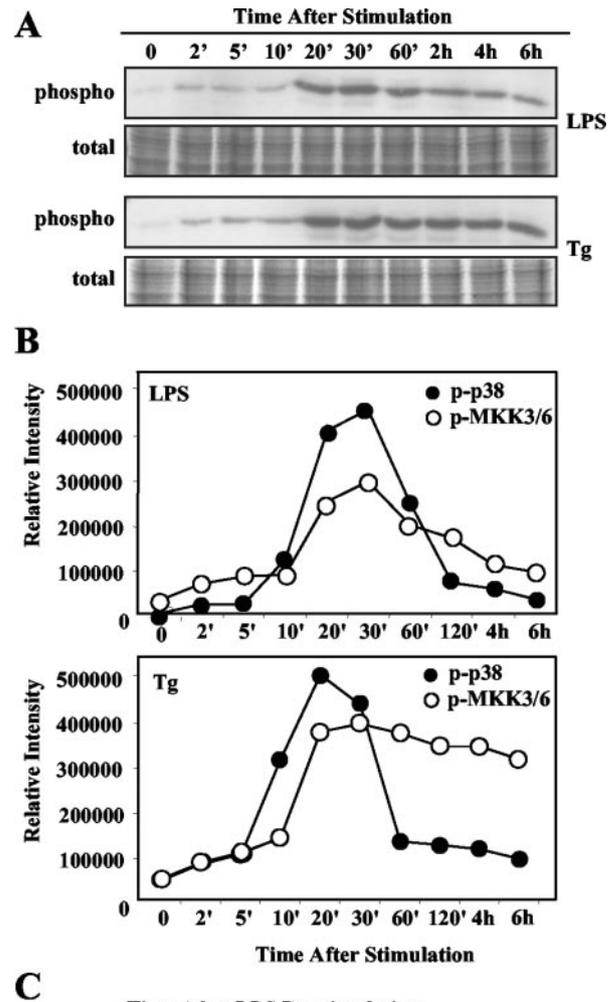
FIGURE 4. LPS and *T. gondii* trigger distinct MKK3/6 activation kinetics. *A*, Mϕ were stimulated with either LPS or *T. gondii* (Tg), and at the indicated time points cell lysates were prepared for immunoblot analysis using phospho-specific MKK3/6 Ab (phospho). In parallel, a gel was stained with Coomassie Blue to assure equivalent protein loading (total). The region shown (37–50 kDa) brackets the molecular mass of MKK3/6 (40 and 41 kDa). Densitometric analysis of phospho-MKK3/6 and phospho-p38 MAPK within each sample was performed on cells stimulated with LPS and *Toxoplasma* (*B*). *C*, Phospho-MKK3/6 was examined in whole cell lysates of Mϕ that were subjected to 6-h pretreatment with medium alone, *Toxoplasma* (Tg; 1:6 ratio of cells to parasites), or LPS (100 ng/ml), followed by secondary LPS stimulation (100 ng/ml) for the indicated times. The experiments were repeated three times with the same result.

decline after 30 min. This kinetic pattern correlated with p38 MAPK activation in the same samples (Fig. 4B). *Toxoplasma* infection also induced MKK3/6 phosphorylation, but interestingly, MKK3/6 activation levels remained elevated for up to 6 h postinfection (Fig. 4A). This contrasted with phospho-p38 MAPK levels in the same samples, which declined to background levels by 2 h postinfection (Fig. 4B). We then examined levels of phospho-MKK3/6 during secondary endotoxin stimulation 6 h after LPS stimulation or *T. gondii* infection (Fig. 4C). As expected, phospho-MKK3/6 levels were already high in parasite-infected cells, but the levels also appeared to increase even further after LPS restimulation. In contrast, phospho-MKK3/6 levels were low in LPS-pretriggered cells, and they were not elevated by secondary endotoxin challenge (Fig. 4C). The results are consistent with findings of others that link endotoxin tolerance with defective signaling early in the LPS-triggered signal transduction cascade (25, 28). The data also demonstrate long term MKK3/6 activation, indicating that defects in phosphorylation of this MKK cannot account for the lack of p38 MAPK activation in *Toxoplasma*-infected cells.

LPS-induced IκBα degradation is maintained in Toxoplasma-preinfected, but not LPS-tolerized, Mφ

We next asked whether the NF-κB activation pathway, which is inactivated during endotoxin tolerance, was also silenced by 6-h *Toxoplasma* pre-infection. As shown in Fig. 5, IκBα degradation, a prerequisite for NF-κB translocation, occurred within 20 min of LPS stimulation, and this was followed by the appearance of newly synthesized IκBα 60 min poststimulation. Consistent with previous reports (33, 34), Mφ that had been prestimulated with LPS failed to undergo IκBα degradation when subjected to secondary LPS triggering (Fig. 5). Infection with *Toxoplasma* also triggered rapid IκBα degradation, followed by its reappearance at 60 min after infection. Interestingly, when Mφ were preinfected with *T. gondii*, IκBα was degraded upon subsequent LPS stimulation, although the kinetics of endotoxin-triggered IκBα degradation in infected cells were delayed compared with those in noninfected Mφ. We do not yet understand the basis for the altered kinetics of IκBα degradation.

We also examined LPS-induced NF-κB translocation in cells pre-exposed to *Toxoplasma* or LPS. Confirming the results showing lack of IκBα degradation, LPS-pretriggered cells failed to translocate NF-κB p65 when subjected to secondary LPS stimu-

lation (Fig. 6A). In contrast, 6-h *T. gondii*-infected Mφ were capable of NF-κB translocation upon subsequent LPS triggering (Fig. 6B). Previously, we showed a p65 translocation blockade in 2-h preinfected Mφ (14), and Fig. 6C reconfirms this result. The results presented in Figs. 5 and 6 combined with those shown in Fig. 4 argue that Mφ reprogramming induced by LPS is distinct from that occurring during *T. gondii* infection.

Toxoplasma *induces long term p38 MAPK inactivation*

Our results show defective MAPK activation, particularly for p38 MAPK, at 6 h postinfection. We determined whether these effects were apparent in long term infected Mφ. For these experiments we employed ts-4, an attenuated RH strain-derived mutant that displays decreased intracellular replication (35). In this manner we avoided nonspecific cytolytic effects associated with use of the rapidly dividing RH strain. As shown in Fig. 7, cells that were infected with ts-4 for 24 h before LPS challenge maintained p38 MAPK nonresponsiveness. Importantly, this was not the case for ERK1/2. In this study the cells displayed only a partial defect in ERK1/2 activation during subsequent LPS triggering. For 24-h LPS-treated Mφ, both p38 MAPK and ERK1/2 activations were
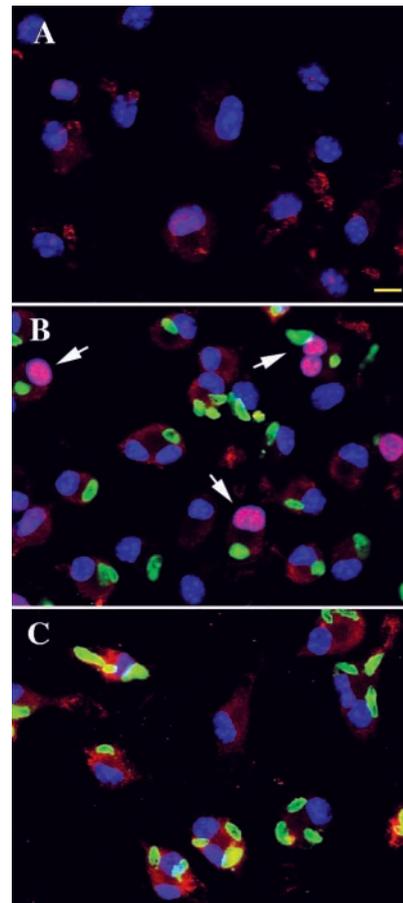

FIGURE 5. LPS-induced IκBα degradation is maintained in *Toxoplasma* pre-infected, but not in LPS-tolerized, Mφ. Cells were subjected to LPS triggering or *Toxoplasma* infection for the indicated times after 6-h preincubation in medium (M+L and M+Tg, respectively). Alternatively, Mφ were pretriggered with LPS (100 ng/ml), then 6 h later were restimulated for the indicated times with LPS (L+L). Similarly, cells were preinfected with *T. gondii* (1:6 ratio of Mφ to parasites), then after 6 h were subjected to LPS stimulation for the times shown (Tg+L). Cell lysates were prepared, and immunoblot analysis was performed with anti-IκBα Ab. This experiment was repeated three times with essentially identical results.

FIGURE 6. Pretriggering Mφ for 6 h with LPS, but not *T. gondii*, prevents NF-κB p65 nuclear translocation upon subsequent LPS stimulation. Bone marrow-derived Mφ were stimulated with LPS (100 ng/ml) for 60 min after 6-h pretreatment with either LPS (A) or *T. gondii* (B). In addition, 60-min LPS secondary stimulation was performed 2 h after *Toxoplasma* infection (C). Cells were subsequently fixed and stained with Ab to p65 (red) and tachyzoite surface Ag-1 (SAG-1; green). Nuclei were subjected to 4′,6-diamidino-2-phenylindole hydrochloride staining (blue). *B*, Arrows point to infected cells undergoing p65 nuclear translocation (appearing as pink nuclei). Yellow bar in *A* = 5 μm. This experiment was performed twice with the same results.
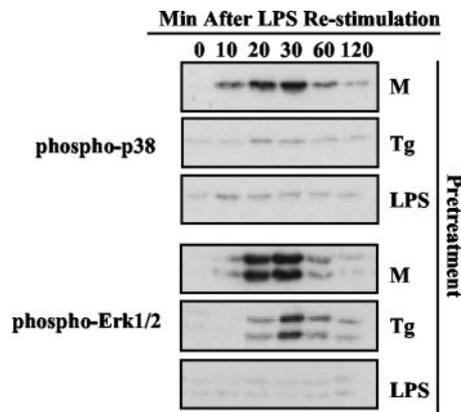
FIGURE 7. Long term infected Mϕ remain p38 MAPK nonresponsive. Cells were precultured in medium (M), preinfected with ts-4 (Tg; 1:6 ratio of Mϕ to parasites), or prestimulated with LPS (100 ng/ml) and 24 h later were subjected to LPS (100 ng/ml) restimulation for the indicated times. MAPK immunoblot analysis was subsequently performed as described in Fig. 3. The experiment was repeated twice with the same results.

defective (Fig. 7). These results are essentially identical with those shown in Fig. 3 for 6-h *Toxoplasma*-infected and LPS-stimulated cells.
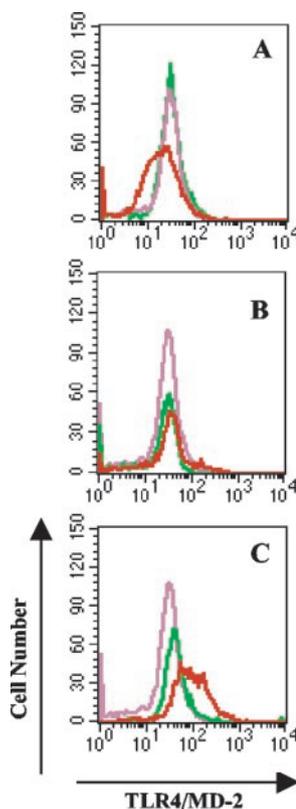
FIGURE 8. Expression of TLR4 is down-regulated by LPS stimulation, but up-regulated by *Toxoplasma* infection. *A*, Mϕ were stimulated with LPS (100 ng/ml), and TLR4/MD-2 expression was examined 6 h (green line) and 24 h (red line) later by flow cytometric analysis relative to that by cells cultured in medium for 24 h (pink line). In parallel cultures, Mϕ were infected with YFP-expressing tachyzoites (3:1 ratio of tachyzoites to cells; parasites from D. Roos, University of Pennsylvania), and TLR4/MD-2 expression 6 h (green line) and 24 h (red line) later was compared with that by unstimulated cells (pink line). In Mϕ exposed to parasites, TLR4/MD-2 expression was examined in noninfected (YFP-negative; *B*) and infected (YFP-positive; *C*) populations. This experiment was repeated twice with the same results.

TLR4 expression is down-regulated by LPS stimulation, but up-regulated by Toxoplasma *infection*

Down-regulation of TLR4, the major sensor of LPS, has been implicated as a mechanism of endotoxin tolerance. Accordingly, we compared the effects of LPS stimulation and *T. gondii* infection on Mϕ TLR4 expression. As shown in Fig. 8*A*, LPS triggering resulted in down-regulation of cell surface TLR4, although this was relatively minor and was not apparent until 24 h after stimulation (mean fluorescence intensity, 16.84 in 24-h LPS-stimulated cells vs 24.14 in medium controls). To examine the effect of *T. gondii* on TLR4 expression, we employed transgenic RH-derived, YFP-expressing tachyzoites. This allowed us to examine TLR4 expression in infected and noninfected Mϕ within the same bulk population. As shown in Fig. 8*B*, there was no change in TLR4 expression in noninfected cells during the course of the experiment. In stark contrast to noninfected and LPS-stimulated Mϕ, tachyzoite-infected cells displayed TLR4 up-regulation that was particularly dramatic 24 h postexposure (Fig. 8*C*; mean fluorescence intensity, 79.95 for 24-h infected cells compared with 30.04 for control noninfected Mϕ).

Discussion

The present studies show that *T. gondii* interferes with signaling pathways that lead to activation of MAPK. Although the parasite stimulated transient activation of p38 MAPK, ERK1/2, and SAPK/JNK, MAPK reactivation was defective when cells were subjected to LPS restimulation. This was most striking for p38 MAPK, which displayed a near-complete lack of phosphorylation when infected cells were subjected to endotoxin triggering. Inasmuch as inhibitor experiments (e.g., Fig. 1) and gene knockout studies implicate p38 MAPK in Mϕ IL-12 and TNF-α production (21), we conclude that blocking this signaling intermediate is one potential way that *Toxoplasma* prevents LPS-triggered cytokine synthesis.

Mϕ that are infected with *Toxoplasma* resemble endotoxin-tolerized cells, because in both cases MAPK activation is disabled. Nevertheless, several lines of evidence demonstrate that the phenomena are distinct. Multiple studies have shown that activation of IL-1R-associated kinase (IRAK) is defective during both homotolerance and heterotolerance to LPS (27, 34, 36). Insofar as IRAK activation is an early signaling event leading to MAPK and NF-κB activation, defective IRAK function provides a potential mechanism for simultaneous inactivation of both NF-κB and MAPK pathways during endotoxin tolerance. For *Toxoplasma* infection, although reactivation of MAPK was defective, degradation of IκBα, an event pivotal in NF-κB activation, continued to occur. This strongly argues against inactivation of upstream signaling intermediates as a mechanism for parasite-induced MAPK inactivation.

Reactivation of p38 MAPK, a kinase important in macrophage TNF-α and IL-12 production (21–24), was severely impaired in LPS-stimulated and *T. gondii*-infected cells. This led us to examine the status of MKK3/6 in each of these situations. For LPS, phosphorylation and dephosphorylation of this kinase paralleled activation and inactivation of p38 MAPK. In an LPS tolerance situation, MKK3/6 failed to undergo reactivation, a result consistent with reprogramming of upstream transduction pathways. Interestingly, during *Toxoplasma* infection MKK3/6 was activated, but failed to undergo deactivation despite the rapid dephosphorylation of p38 MAPK. Little is known regarding inactivation of MKK, but the simplest explanation for these data is that a MKK phosphatase acting on MKK3/6 is induced by endotoxin stimulation, but not *Toxoplasma* infection. In this regard, the serine/threonine protein phosphatase type 2Cα has been shown to inhibit p38

MAPK and SAPK/JNK signaling cascades by dephosphorylating MKK6, MKK4, as well as p38 MAPK itself (37).

MAPK deactivation is tightly controlled by a family of dual-specificity MAPK phosphatases (38). The prototypic member is MKP-1, which preferentially targets p38 MAPK and SAPK/JNK (20, 32). As shown in this study, LPS-triggered MKP-1 induction correlates precisely with deactivation of these MAPK. It has also recently been demonstrated that ectopic MKP-1 expression accelerates inactivation of p38 MAPK and SAPK/JNK (20). Because induction of MKP-1 is itself dependent upon ERK1/2, this forms a potent negative feedback loop that regulates MAPK-dependent responses. *Toxoplasma* infection failed to elicit MKP-1, yet after early activation, p38 MAPK and SAPK/JNK underwent prompt deactivation. This result suggests that MAPK phosphatases other than MKP-1 control deactivation during intracellular *T. gondii* infection. Alternate candidates would be MKP-5 and M3/6 (hVH5), which also display selectivity for p38 MAPK and SAPK/JNK (39–41). We are currently examining in further detail MAPK phosphatase activity during *Toxoplasma* infection.

We also found that in 6-h *T. gondii*-infected cells, elevated phospho-MKK3/6 levels were maintained and were increased further in response to LPS. The results suggest that for p38 MAPK, defective LPS-mediated activation in *Toxoplasma*-infected cells does not result from defects in upstream signaling, as appears to be the case during endotoxin tolerance. Instead, our data suggest that downstream events, such as induction of MAPK phosphatase activity, may be responsible for parasite-induced nonresponsiveness to endotoxin challenge. Nevertheless, it is possible that *Toxoplasma* interferes with the activation of another MKK that phosphorylates p38 MAPK. For example, MKK4, an upstream SAPK/JNK kinase, has also been implicated in p38 MAPK activation (42–44). Further studies are required to resolve these issues.

Related to our experiments are those of others that implicate *Trypanosoma cruzi* mucin-like glycoproteins in the induction of heterotolerance to LPS (19). In this case, p38/SAPK2-dependent induction of a protein phosphatase type 2A was suggested to be involved in the defective IRAK-1 phosphorylation occurring during induction of tolerance by *T. cruzi* glycoproteins. During *Leishmania* infection, defective Janus kinase 2 phosphorylation has been linked to activation of the cytoplasmic protein tyrosine phosphatase Src homology protein 1 (45). Thus, it seems likely that the hijacking of phosphatase-dependent pathways that regulate protein kinase cascades is an effective general strategy employed by microbial pathogens to manipulate host responses during intracellular infection.

Down-regulation of TLR4 has been suggested as a mechanism for endotoxin tolerance, although the physiological significance of this has been debated (25, 29). In our hands, we found that LPS stimulation did indeed induce TLR4 down-regulation. However, the effect was not apparent until 24 h post-triggering. At 6 h, when we performed our LPS retriggering experiments and found defective MAPK activation, TLR4 expression was normal. The effects of *T. gondii* on TLR4 expression were distinct from those of LPS. In this study intracellular infection resulted in TLR4 up-regulation. We conclude from our results that down-regulation of TLR4 cannot account for MAPK nonresponsiveness during LPS stimulation or *Toxoplasma* infection.

Our demonstration that *Toxoplasma* induces transient MAPK activation with kinetics similar to those of LPS raises the question of why infection fails to elicit early cytokine production as occurs during endotoxin stimulation. The reasons for this are not clear, but may relate to recent observations that nuclear translocation of NF-κB is temporarily blocked by *T. gondii* (14, 15, 46). Thus, during infection, MAPK- and NF-κB-dependent signaling is initiated, but in the case of NF-κB and possibly MAPK, it cannot be completed because of defects in nuclear translocation. The initial blockade is eventually removed, but at later time points defects in MAPK activation prevent LPS-triggered TNF-α and IL-12 production. Molecular identification of parasite molecules and mechanisms that interfere with MAPK signaling pathways will provide insight into host-pathogen interactions during intracellular infection and may lead to identification of anti-inflammatory molecules of clinical importance.

Acknowledgments

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