View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Cell, Vol. 103, 1071–1083, December 22, 2000, Copyright ©2000 by Cell Press

TNF-α Induction by LPS Is Regulated Posttranscriptionally via a Tpl2/ERK-Dependent Pathway

Calin D. Dumitru,[1] Jeffrey D. Ceci,[2,5,8]
Christos Tsatsanis,[1,6,8] Dimitris Kontoyiannis,[3,8]
Konstantinos Stamatakis,[1] Jun-Hsiang Lin,[1]
Christos Patriotis,[7] Nancy A. Jenkins,[2]
Neal G. Copeland,[2] George Kollias,[3]
and Philip N. Tsichlis[1,4]
[1] Kimmel Cancer Center
Department of Microbiology and Immunology
Thomas Jefferson University
233 S. 10th Street
Philadelphia, Pennsylvania 19107
[2] Mouse Cancer Genetics Program
National Cancer Institute—FCRDC
Frederick, Maryland 21702
[3] Laboratory of Molecular Genetics
Hellenic Pasteur Institute
Athens 115 21
Greece

Summary

***Tpl*2 knockout mice produce low levels of TNF-α when exposed to lipopolysaccharide (LPS) and they are resistant to LPS/D-Galactosamine-induced pathology. LPS stimulation of peritoneal macrophages from these mice did not activate MEK1, ERK1, and ERK2 but did activate JNK, p38 MAPK, and NF-κB. The block in ERK1 and ERK2 activation was causally linked to the defect in TNF-α induction by experiments showing that normal murine macrophages treated with the MEK inhibitor PD98059 exhibit a similar defect. Deletion of the AU-rich motif in the TNF-α mRNA minimized the effect of *Tpl2* inactivation on the induction of TNF-α. Subcellular fractionation of LPS-stimulated macrophages revealed that LPS signals transduced by Tpl2 specifically promote the transport of TNF-α mRNA from the nucleus to the cytoplasm.**

Introduction

Protection from microbial pathogens is mediated by a variety of inducible effector mechanisms that are triggered by these microbes. The sum of these mechanisms defines antimicrobial immunity, which is subclassified into innate and adaptive (for review, see Medzhitov and Janeway, 1997a). Innate immunity appeared early in evolution and can be recognized to date in both vertebrate and invertebrate species (for review, see Medzhitov and Janeway, 1997a, 1997b). Innate immunity is triggered by pathogen-associated molecular patterns (PAMPs) that are shared by groups of microbial pathogens and that are recognized by pattern recognition receptors (PRRs) in host cells. PRRs in turn include signaling and endocytic receptors, as well as secreted proteins that bind the microbes and facilitate their phagocytosis or destruction by the complement system (Medzhitov and Janeway, 1997b).

Pathogen-associated molecular patterns are represented by molecules localized on microbial walls. Prominent among them is lipopolysaccharide (LPS) or endotoxin, which is derived from Gram-negative bacteria. LPS binds the soluble LPS binding protein (LBP) and the complex binds CD14, a monocyte/macrophage receptor molecule that is expressed in both soluble and membrane-associated forms (for review see Ulevitch and Tobias, 1995). CD14 presents the LPS-LBP complex to the LPS receptor TLR4, a member of the Toll family of receptors originally identified in *Drosophila* (Medzhitov et al., 1997). TLR4 is the signaling receptor and responds to LPS even in the absence of CD14, whose role is to form, in combination with TLR4, a high-affinity receptor. A mutation in TLR4 that arose spontaneously in C3H/HeJ mice (P712H) rendered these mice LPS-unresponsive (Poltorak et al., 1998).

Signals originating in the LPS-triggered TLR4 receptor activate several signaling pathways in target cells such as B cells and macrophages (Schletter et al., 1995). Molecules produced by cells responding to LPS target pathogens directly or indirectly. Although these biological responses protect the host against invading pathogens, they may also cause harm. Thus, massive stimulation of innate immunity, occurring as a result of severe Gram-negative bacterial infections, leads to excess production of cytokines and other molecules, and the development of a fatal syndrome, the septic shock syndrome, which is characterized by fever, hypotension, disseminated intravascular coagulation, and multiple organ failure (Parillo, 1993).

There are two mouse models for the septic shock syndrome. In the first model, the syndrome is induced by the administration of a large dose of LPS and is characterized by the development of symptoms over a period of several days to a week (Fink and Heard, 1990). Mice deficient in ICE (Li et al., 1995) or caspase-11 (Wang et al., 1998), which are required for the processing of IL-1β, are also required for susceptibility to high dose LPS. Mice injected intravenously (IV) with anti-IL-1 neutralizing antibodies or antagonists of IL-1 become resistant to high dose LPS-induced shock (reviewed in Dinarello et al., 1993), suggesting that IL-1 is the main cytokine responsible for this syndrome. In agreement with this, ICE and caspase-11 are also required for susceptibility to high dose LPS (Li et al., 1995; Wang et al., 1998). In the second model, the syndrome is induced by the administration of a low dose of LPS combined with the transcriptional inhibitor D-Galactosamine (Galanos et

[4] To whom correspondence should be addressed (e-mail: p_tsichlis@lac.jci.tju.edu).
[5] Present address: Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555.
[6] Present address: Clinical Chemistry Laboratory University of Crete Medical School, 71409 Heraklion, Crete.
[7] Present address: Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111.
[8] J. D. C. generated the *Tpl2* −/− mice, C. T. first observed the resistance of *Tpl2* −/− to LPS, and D. K. performed the experiments with the *TNF-α* ΔARE/− mice.

al., 1979). This gives rise to a rapidly developing syndrome that culminates in death within 6–10 hr. Mice deficient in TNF-α (Pasparakis et al., 1996) or TNFR1 (Pfeffer et al., 1993) are resistant to LPS/D-Galactosamine-induced shock, suggesting that the main cytokine responsible for this syndrome is TNF-α. The induction of TNF-α by LPS in macrophages, the main cellular effector in LPS/D-Galactosamine-induced shock, is mediated by both transcriptional and posttranscriptional mechanisms (Raabe et al., 1998). Particularly important in the regulation of TNF-α is an AU-rich element (ARE) in the 3′-untranslated region of the TNF-α mRNA that represses TNF-α expression posttranscriptionally. LPS signals transduced via the JNK and p38 MAPK pathways relieve the ARE-dependent posttranscriptional repression (Kontoyiannis et al., 1999). As a result, inhibition of the JNK pathway inhibits TNF-α production by macrophages. Moreover, inactivation of MAPKAP Kinase 2, a target of the p38 MAPK pathway, renders mice resistant to LPS/D-Galactosamine-induced shock (Kotlyarov et al., 1999).

The *Tpl2* protooncogene, also known as *Cot*, encodes a serine threonine protein kinase that is activated by provirus integration in MoMuLV-induced rodent T cell lymphomas and MMTV-induced mammary carcinomas (Patriotis et al., 1993; Erny et al., 1996; Ceci et al., 1997). Provirus integration always occurs in the last intron of the gene and gives rise to mRNA transcripts that encode a carboxy-terminally truncated kinase that is constitutively active and highly oncogenic. Transgenic mice expressing the truncated form of Tpl2 under the control of a T cell-specific promoter develop T cell lymphoblastic lymphomas by the age of 3 months (Ceci et al., 1997). Earlier studies revealed that overexpression of Tpl2 activates the ERK, JNK, and p38 MAPK pathways (Patriotis et al., 1994; Salmeron et al., 1996; Chiariello et al., 2000) as well as the transcription factors NFAT and NF-κB (Tsatsanis et al., 1998a, 1998b; Belich et al., 1999; Lin et al., 1999).

In this report, we show that *Tpl2* knockout mice are resistant to LPS/D-Galactosamine-induced endotoxin shock and that their resistance is due to a posttranscriptional defect in the induction of TNF-α by LPS. Moreover, we show that Tpl2 is required for the activation of ERK1 and ERK2 by LPS and that the ERK pathway is obligatory for the posttranscriptional regulation of TNF-α. Finally, we present evidence that the Tpl2/ERK-transduced signals that specifically control TNF-α induction by LPS regulate nucleocytoplasmic mRNA transport by a mechanism that targets the AU-rich element in the 3′UTR of the TNF-α RNA.

Results

Establishment and Initial Characterization of *Tpl2* Knockout Mice

To determine the physiological role of Tpl2 at the animal level, we generated *Tpl2* knockout mice by disrupting the *Tpl2* gene in ES cells via homologous recombination (Figures 1A and 1B). The *Tpl2* targeting construct contained the G418-resistance gene flanked by genomic sequences derived from exons 3 and 5 (Figure 1A). Homologous recombination between the *Tpl2* sequences in the construct and the genomic *Tpl2* DNA deleted a portion of the *Tpl2* catalytic domain including the ATP binding site and the activation loop. Injection of ES cell clones carrying the disrupted *Tpl2* gene into blastocysts derived from C57BL/6 mice gave rise to *Tpl2* +/−/ *Tpl2* +/+ chimeras. The chimeras were backcrossed to C57BL/6. Mice utilized in the experiments reported here were derived by brother-sister mating of *Tpl2* −/− or *Tpl2* +/+ littermates obtained after nine consecutive backcrosses to C57BL/6. Splenocytes from *Tpl2* knockout mice do not express Tpl2 (Figure 1C). The lack of detection of a defective *Tpl2* mRNA in the *Tpl2* −/− spleens may be due to the fact that such an RNA may be unstable. Alternatively, transcription may be impaired.

The *Tpl2* knockout mice developed normally and did not exhibit obvious phenotypic defects. Given that *Tpl2* is primarily expressed in hematopoietic tissues, these mice were further analyzed with regard to their hematologic and immunologic phenotype. The results showed that the bone marrow, thymus, spleen, and lymph nodes are histologically normal (Figure 1D) and that they contain all the expected cell subsets at the normal ratio. Specifically, the spleens and thymuses of *Tpl2* −/− and *Tpl2* +/+ mice were shown to contain the same percentage of cells expressing all the combinations of CD4/CD8 or B220/Thy1.2 markers. Moreover, the spleens and thymuses of the *Tpl2* knockout and control mice contained the same overall percentage of cells carrying the markers CD4, CD8, Thy1.2, B220, Mac-1, Ter-1, CD3, IL-2Rα, TCR$\alpha\beta$, and TCR$\gamma\delta$ (data not shown). The *Tpl2* knockout mice also showed normal antibody responses to the T cell-dependent antigen KLH and to the T cell-independent antigen LPS-TNP. Following inoculation with lymphocytic choriomeningitis virus (LCMV), they showed normal T cell-mediated cytotoxic responses against LCMV-infected cells (data not shown). Finally anti-CD3 plus anti-CD28-stimulated splenocytes from 8-week-old *Tpl2* −/− and *Tpl2* +/+ mice produced similar levels of cytokines including IL-2, TNF-α, IL-4, and IFNγ and incorporated similar amounts of ^{3}H-thymidine (data not shown).

Tpl2 Knockout Mice Secrete Low Levels of TNF-α in Response to LPS and They Are Resistant to Endotoxin Shock

To determine whether Tpl2 has a role in the response to inflammatory signals, *Tpl2* −/− and *Tpl2* +/+ mice were inoculated intraperitoneally (IP) with 25 mg D-Galactosamine plus increasing doses of *Salmonella enteritidis*-derived lipopolysaccharide (LPS). D-Galactosamine is a hepatotoxic transcriptional inhibitor, which sensitizes the animals to the cytotoxic effects of TNF-α (Leist et al., 1994). The results (Figure 2A) revealed that *Tpl2* knockout mice are resistant to the induction of endotoxin shock. Thus, while all the *Tpl2* +/+ mice died within 9 hr following injection of LPS, the majority of the *Tpl2* knockout mice survived. The surviving *Tpl2* −/− mice were followed for a period of 7 days without any evidence of late occurring ill effects.

The syndrome arising in LPS/D-Galactosamine-treated mice is characterized by hepatocyte apoptosis and depends on the action of TNF-α (Pfeffer et al., 1993; Pasparakis et al., 1996). We therefore examined whether

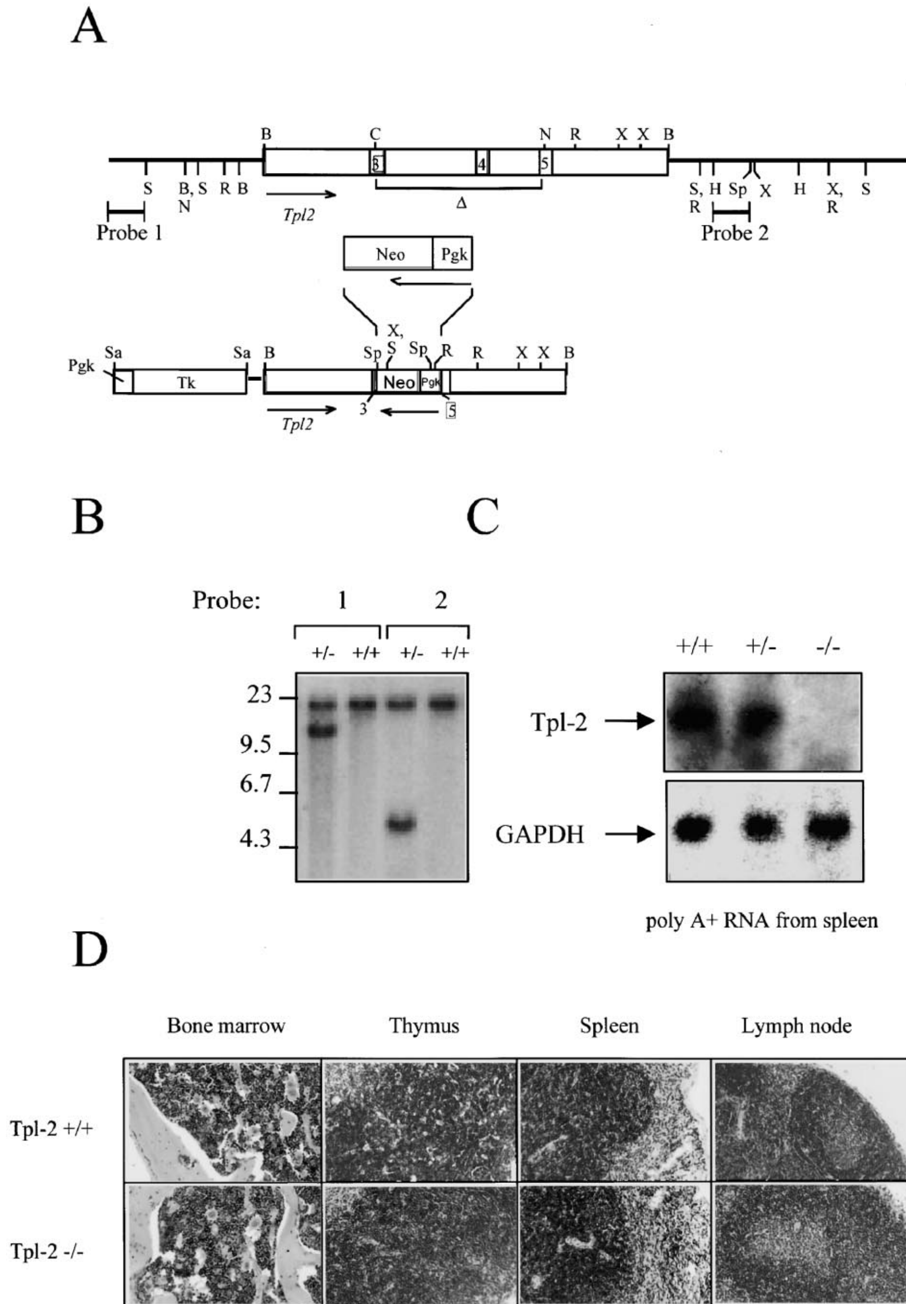


Figure 1. Establishment of the *Tpl-2* −/− Mice

(A) The *Tpl2* targeting construct*.* The upper panel shows the restriction map of a *Tpl2* genomic clone isolated from a 129/Sv mouse genomic DNA library. The code for the restriction enzymes in the map is as follows: B, BamHI, C-ClaI, H-HindIII, N-NheI, R-EcoRI, S-SacI, Sa-SalI, Sp-SphI, X-XbaI. The boxed BamHI-BamHI DNA fragment includes the sequences BamHI-ClaI and NheI-BamHI, which were cloned into the targeting vector 5′ and 3′ of the G418 resistance gene, respectively, and the *Tpl2* sequences that were replaced by the G418 resistance cassette via homologous recombination. The lower panel shows the structure of the *Tpl2* targeting construct.

(B) Screening for homologous recombination between the vector and the endogenouts *Tpl2* gene*.* ES cell genomic DNA was digested with SphI. Following electrophoresis and transfer, it was hybridized to the probes 1 and 2 shown in A.

(C) *Tpl-2* −/− mice do not express Tpl-2. Northern blotting analysis of poly-A+ mRNA extracted from spleens of *Tpl-2* −/−, *Tpl-2* +/−, and *Tpl-2* +/+ mice. The blot was hybridized to a 1.5 kb PstI-PstI fragment retrieved from the Tpl2 cDNA (Ceci et al., 1997). Equal loading was confirmed by hybridizing the same blot to a GAPDH probe.

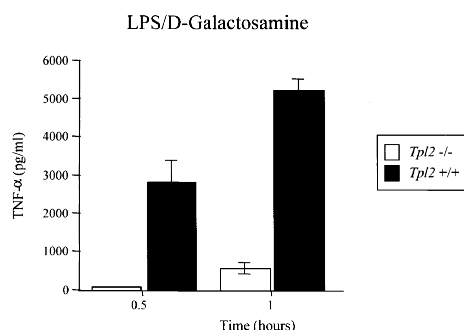
(D) Hematoxyllin and eosin stained paraffin sections of bone marrow, thymus, spleen, and lymph nodes from *Tpl2* +/+ and *Tpl2* −/− mice.

Tpl2 −/− mice secrete TNF-α in response to LPS. The results showed that while TNF-α was detected in the serum of control mice at 30 min and at 1 hr following LPS stimulation, it could barely be detected in the serum of *Tpl2* −/− mice (Figure 2B). IL-1β was induced in *Tpl2* −/−, although at levels lower than those in control mice

A

Genotype	Amount of LPS injected (in mg)	Death at 9 hours
Tpl-2 +/+	0.1	5/5
	1	3/3
	10	9/9
Tpl-2 -/-	0.1	1/7
	1	0/3
	10	2/9
Tpl-2 +/-	1	3/3

B



C

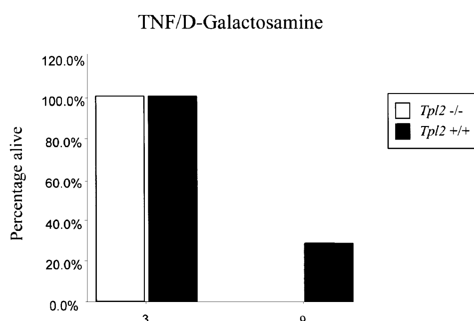


Figure 2. The Resistance of *Tpl2* −/− Mice to LPS/D-Galactosmine-Induced Endotoxin Shock Is Due to a Defect in TNF-α Secretion in Response to LPS

(A) *Tpl-2* −/− mice are resistant to LPS/D-galactosamine-induced endotoxin shock. 8- to 12-week-old mice were injected IP with D-glactosamine (1 mg/g body weight) and with the indicated amounts of LPS (from *Salmonella enteritidis*, Sigma L-6011).

(B) LPS-induced TNF-α secretion is impaired in *Tpl2* −/− mice. *Tpl2* −/− and *Tpl2* +/+ mice were injected IP with D-galactosamine and LPS from *Salmonella enteritidis*. Half an hour and 1 hr later, sera samples were collected from the tail vein. TNF-α levels were determined by ELISA (Pharmingen). Data are shown as mean value ± standard deviation.

(C) *Tpl2* −/− mice are sensitive to TNF-α -induced toxicity. Six *Tpl2* +/+ mice and 10 *Tpl2* −/− mice (8 to 12 weeks old) were injected with D-Galactosamine IP and, 1 hr later, with 35 ng/g body weight murine TNF-α IV. Time of death was recorded starting from the time of injection of TNF-α.

(data not shown). In agreement with this data, *Tpl2* −/− mice were also resistant to high dose LPS-induced shock (data not shown). To verify that the LPS resistance phenotype is due to the observed defect in TNF-α induction and not to a defect in TNF-α signaling, *Tpl2* knockout and control mice were injected with 25 mg D-Galactosamine IP and with 1.2 μg TNF-α IV. The results showed that both the *Tpl2* knockout and the control mice are TNF-α sensitive (Figure 2C).

Splenocytes, Thioglycollate-Elicited Peritoneal Macrophages (TEPM), and Bone Marrow–Derived Macrophages (BMDM) from Tpl2 Knockout Mice Are Defective in TNF-α Induction by LPS

Short-term cultures of unfractionated splenocytes, TEPM, and BMDM were stimulated with LPS. Culture supernatants were collected at the indicated time points and assayed for TNF-α using an ELISA assay. Figures 3A and 3B show that both splenocytes and macrophages from *Tpl2* knockout mice were defective in TNF-α secretion in response to LPS. This is not due to a general impairment in cytokine release because at least one cytokine (IL-12) was overproduced by the *Tpl2* −/− macrophages by comparison with the *Tpl2* +/+ macrophage controls (data not shown). The low responsiveness of the Tpl2 deficient cells was specific for LPS in that splenocytes from *Tpl2* −/− and control animals secreted equivalent levels of TNF-α when stimulated with anti-CD3 plus anti-CD28 (Figure 3A) or PMA plus ionomycin (data not shown). Moreover, the low responsiveness to LPS was specific for TNF-α induction in that LPS-induced proliferation of splenocytes was equivalent in cells derived from wild-type and *Tpl2* −/− mice (Figure 3A, right panel).

To determine whether it was the secretion or the synthesis of TNF-α that was defective in cells derived from *Tpl2* −/− mice, peritoneal macrophages were treated with LPS plus monensin, which inhibits the transport of proteins from the Golgi apparatus (Uchida et al., 1980). Six hours later, the cells were permeabilized with saponin and they were stained with FITC-labeled anti-TNF-α antibody. Stained cells were analyzed by flow cytometry. The results (Figure 3C, left panel) showed that macrophages from *Tpl2* −/− mice are defective in TNF-α synthesis. To confirm that the defect in TNF-α synthesis was the direct result of the *Tpl2* inactivation, Tpl2 reconstituted and nonreconstituted *Tpl2* −/− BMDM were similarly analyzed for TNF-α induction by LPS. The results (Figure 3C, right panel) showed that reconstitution corrects the defect in TNF-α synthesis and confirmed that Tpl-2 directly regulated the induction of TNF-α.

The finding that the defect in TNF-α synthesis in *Tpl2* −/− macrophages is LPS specific suggests that Tpl2 functions at the top of the cascade of TNF-α induction prior to its convergence with TNF-α induction cascades initiated by other stimuli. However, it is unlikely that Tpl2 functions at the level of the LPS receptor because *Tpl2* −/− mice secrete nitric oxide in response to LPS (data not shown) and splenic B cells from *Tpl2* −/− mice proliferate equally well with splenic B cells from *Tpl2* +/+ mice following LPS stimulation (Figure 3A, right panel). To confirm this conclusion, we examined the expression of CD14, the macrophage high-affinity receptor, at the surface of TEPM from *Tpl2* −/− and *Tpl2* +/+ mice. The results revealed that peritoneal macrophages from *Tpl2* knockout and control mice express similar levels of CD14 (Figure 3D). We therefore conclude that Tpl2 does not function at the level of the LPS receptor.

The Defect in TNF-α Induction in Response to LPS in *Tpl2* −/− Mice Is Posttranscriptional

To define the mechanism by which Tpl2 transduces LPS signals and regulates TNF-α induction, we first exam-

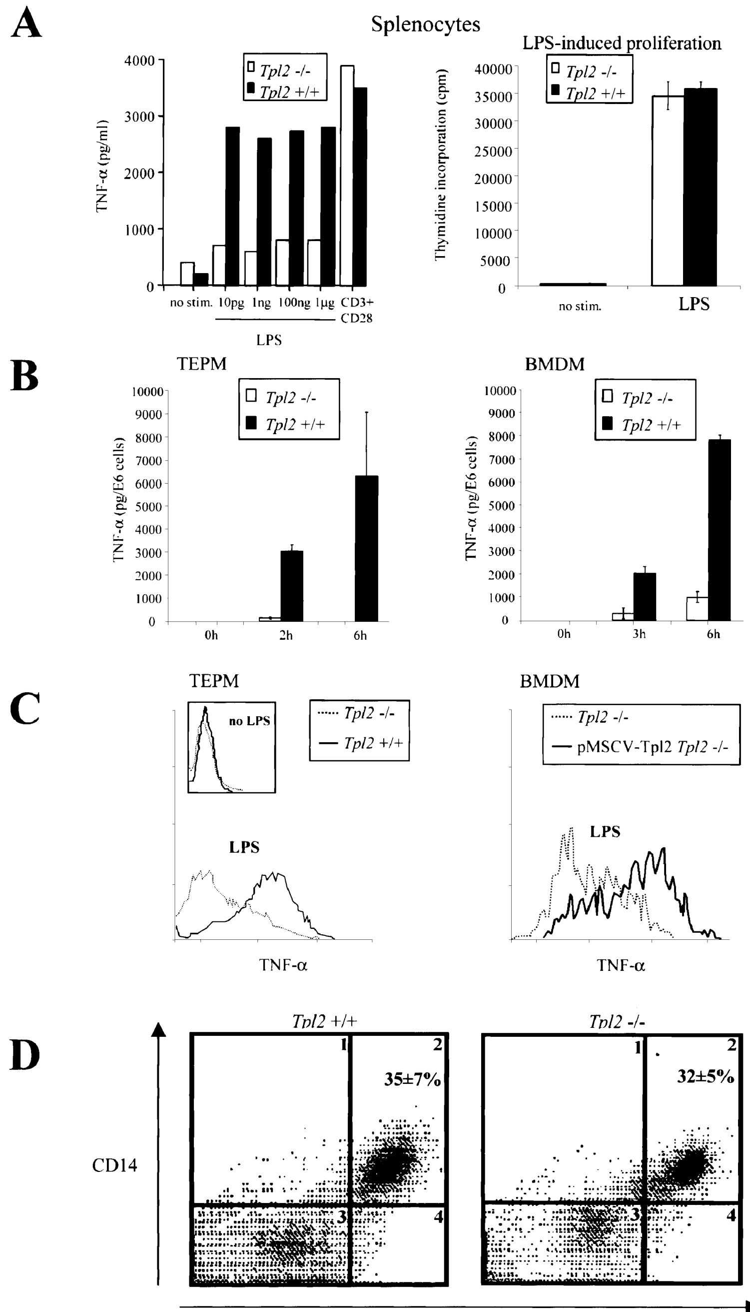


Figure 3. Splenocytes, TEPM, and BMDM from *Tpl2* −/− Mice Are Defective in TNF-α Production in Response to LPS. Expression of the High-Affinity LPS Receptor in TEPM

(A) Left Panel: unfractionated splenocytes were distributed in 96 well plates (5×10^5 cells/well in 200 μl). The cells were treated with LPS from *Salmonella enteritidis* at the concentrations shown in the figure. Alternatively, the cells were exposed to anti-CD3 plus anti-CD28 antibodies. Supernatants were collected after 24 hr of incubation and the TNF-α titers were measured by ELISA. Right Panel: unfractionated splenocytes were stimulated with 1μg/ml LPS. 24 hr later they were pulsed with ^{3}H-thymidine (0.1 μCi/well).

(B) TEPM and BMDM from three *Tpl2* +/+ and three *Tpl2* −/− mice were distributed into 24 well plates (10^6 cells/ml). Attached cells were washed twice with DMEM supplemented with 0.5% FBS and they were cultured in the same medium overnight. 16 hr later they were placed back into 10% FBS-containing medium with or without LPS (1 μg/ml final concentration). Culture supernatants were collected at different time points and the amount of secreted TNF-α was measured by ELISA (Pharmingen) in both the LPS-treated and untreated samples.

(C) Left panel: TEPM from *Tpl2* +/+ and *Tpl2* −/− mice were incubated for 6 hr with LPS (1 μg/ml) and with monensin (2 μM) (GolgiStop, Pharmingen). Cells harvested by scraping were washed, permeabilized with 0.1% saponin, and stained for intracellular TNF-α with FITC-labeled anti-TNF-α antibody. Right panel: *Tpl2* +/+ BMDM were reconstituted by infection with a high titer retrovirus expressing Tpl2 wt (pMSCV-Tpl2) as described in Experimental Procedures. The cells were stimulated with LPS in the presence of monensin and TNF-α expression was measured 4 hr later in Tpl2-expressing and nonexpressing cells.

(D) TEPM recovered by washing the peritoneal cavity of thioglycollate-treated mice with cold HBSS were stained with anti-Mac-1 antibody from Pharmingen which recognizes a molecule specifically expressed in macrophages and with anti-CD14 (Pharmingen). Stained cells were identified by flow-cytometry.

ined whether the inability of *Tpl2* −/− mice to produce normal levels of TNF-α in response to LPS is due to a transcriptional or posttranscriptional defect. To this end, *Tpl2* −/− and *Tpl2* +/+ mice were inoculated with 1 μg LPS IP. RNA from the spleens of the inoculated animals, harvested 1 hr later, was analyzed for TNF-α induction by Northern blotting or by RNase protection. The results (Figures 4A1 and 4A2) showed that the induction of TNF-α mRNA is equivalent in spleens of *Tpl2* −/− and *Tpl2* +/+ mice. In parallel experiments, TEPM and BMDM from *Tpl2* −/− and *Tpl2* +/+ mice were placed in culture (1×10^6 cells/ml) and expression of TNF-α was examined by RNase protection before and 1 hr after stimulation with LPS. The results of this experiment (Figures 4B1 and 4B2) were similar to the results of the in vivo experiment in that they also showed equivalent induction of TNF-α mRNA in *Tpl2* −/− and *Tpl2* +/+ macrophages.

Tpl2 Is Required for the Activation of ERK1 and ERK2 but Not ERK5 by LPS

When overexpressed in a variety of cell types, Tpl2 activates the ERK, JNK, and p38 MAPK pathways, NFAT and NF-κB (Patriotis et al., 1994; Salmeron et al., 1996; Ceci et al., 1997; Tsatsanis et al., 1998a, 1998b; Belich et al., 1999; Lin et al., 1999; Chiariello et al., 2000). Interestingly, LPS activates the same pathways (Schletter et al., 1995). Since Tpl2 functions downstream from the LPS receptor but at the top of the TNF-α induction cas-

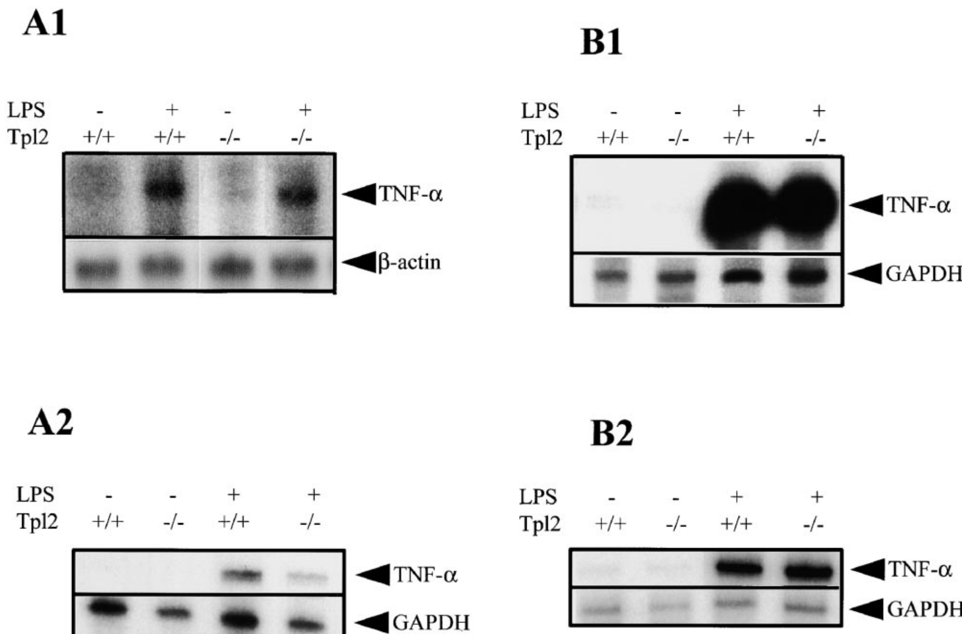


Figure 4. Induction of TNF-α mRNA by LPS In Vivo and In Culture. The Defect in TNF-α Induction in Response to LPS in *Tpl2* −/− Mice Is Posttranscriptional

(A1) *Tpl2* +/+ and *Tpl2* −/− mice were inoculated with D-Galactosamine and LPS and were sacrificed 1 hr later. 12 μg of total spleen RNA from LPS-injected and uninjected control mice were Northern blotted and hybridized to TNF-α and β-actin cDNA probes. Hybridizing bands were visualized by autoradiography and quantitated by PhosphorImager scanning.

(A2) The levels of TNF-α mRNA in the same spleen RNA samples was determined by RNase protection with GAPDH used as a loading control. The TNF-α/GAPDH ratio was measured by PhosphorImager and it was found to be the same in *Tpl2* −/− and *Tpl2* +/+ spleens.

(B1 and B2) TNF-α mRNA levels in TEPM (B1) and BMDM (B2) as determined by RNase protection*.* GAPDH was used as a loading control. All cells were cultured in DMEM supplemented with 0.5% FBS overnight prior to their exposure to LPS (1 μg/ml) for 1 hr.

cade, it may contribute to the activation of all these pathways. However, due to redundancy in cellular signaling, it may be required for the activation of only some. Pathways that depend on Tpl2 may be obligatory for TNF-α induction by LPS.

To address these questions, we examined the activity of MEK1, MEK2, ERK1, ERK2, JNK1, JNK2, and p38 MAPK in LPS-stimulated TEPM from *Tpl2* −/− and *Tpl2* +/+ mice before and after stimulation with LPS. Kinase activation was determined both by Western blotting using antibodies that specifically recognize the phosphorylated, activated forms of these kinases as well as by immunocomplex in vitro kinase assays. The results (Figures 5Aa, 5Ac, 5Ba, and 5Bc) revealed that the inactivation of *Tpl2* specifically blocks the activation of ERK1 and ERK2. Identical results were obtained with bone marrow–derived macrophages (data not shown). Since recent studies showed that Tpl2 activates ERK5 when expression constructs of both are cotransfected into NIH3T3 cells (Chiariello et al., 2000), we examined whether LPS activates ERK5 in primary *Tpl2* +/+ and *Tpl2* −/− BMDM. The results showed that although sorbitol (positive control) activated ERK5 in both types of macrophages, LPS does not activate it in either (Figure 5Ad).

To determine whether the block in the activation of ERK1 and ERK2 by LPS is responsible for the defect in TNF-α induction, we stimulated peritoneal macrophages from normal mice with LPS before and after treatment with the MEK inhibitor PD98059 (Dudley et al., 1995). Supernatants of these cultures harvested 2 hr later were analyzed by ELISA for TNF-α secretion. Moreover, cell lysates were analyzed for ERK1 and ERK2 activation. The results showed that when the activation of ERK1 and ERK2 is blocked, the induction of TNF-α is also blocked (Figures 5C1 and 5C2). Activation of ERK1 and ERK2 is therefore obligatory for TNF-α induction by LPS. The same experiment was repeated in bone marrow macrophages with similar results (data not shown).

Tpl2 Is Not Required for LPS-Induced Activation of NF-κB. *NK-κB1* Knockout Mice Are Susceptible to LPS/D-Galactosamine-Induced Endotoxin Shock

To determine whether Tpl2 is required for the activation of NF-κB, we examined NF-κB DNA binding activity in nuclear extracts of peritoneal macrophages before and 60 min after LPS stimulation. The results in Figure 5D1 show that NF-κB was induced equally well in *Tpl2* −/− and *Tpl2* +/+ cells. In agreement with these data, there was no difference in IKKα and β activation in by LPS (data not shown).

Since recent studies reported that Tpl2 plays a critical role in the processing of p105 (NF-κB1) (Belich et al., 1999), we examined the susceptibility of *NF-κB1* knockout mice (Sha et al., 1995) to LPS/D-Galactosamine-induced endotoxin shock. The results showed that *NF-κB1* knockout mice are susceptible to shock (Figure 5D2) and excluded NF-κB1 as an important determinant of the described *Tpl2* knockout phenotype.

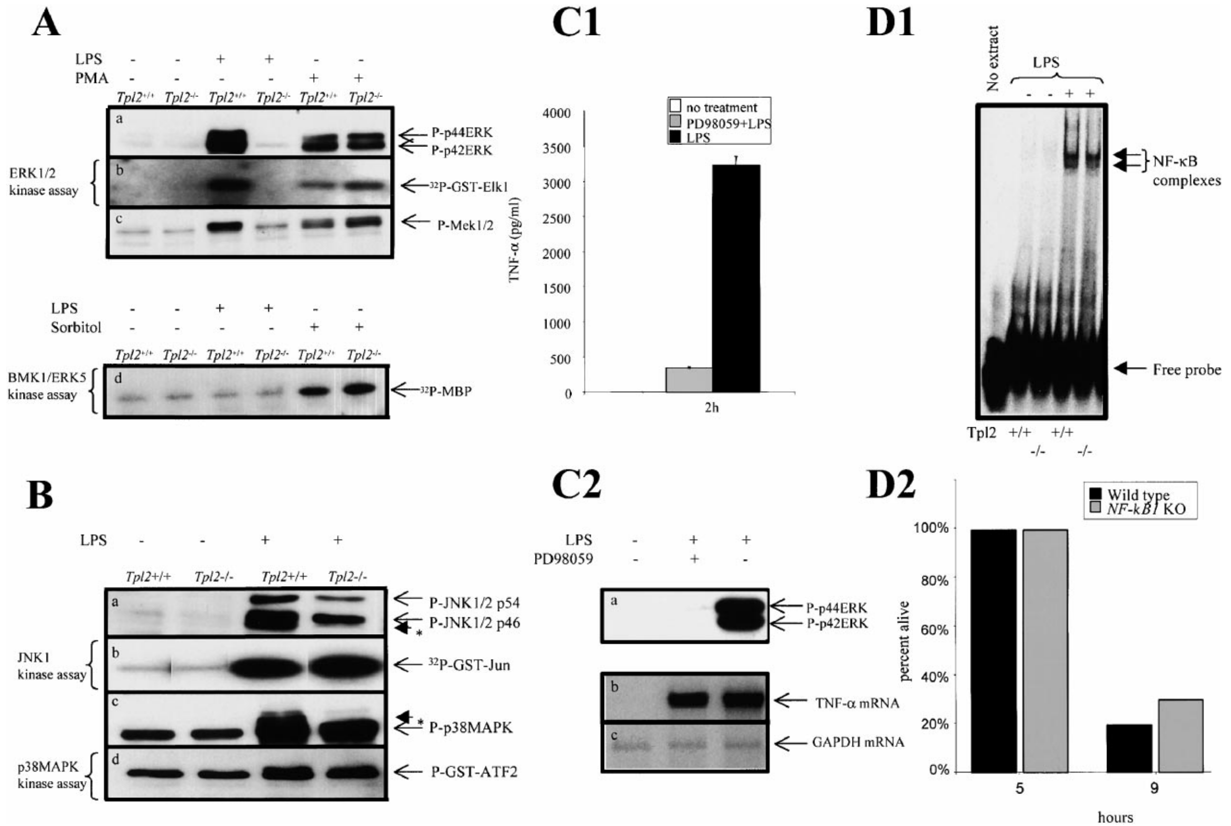


Figure 5. Tpl2 Is Required for the Activation of the ERK Pathway by LPS. Activation of the ERK Pathway Is Required for the Induction of TNF-α by LPS. Tpl2 Is Not Required for LPS-Induced Activation of NF-κB; NF-κB1 −/− Mice Are Sensitive to LPS/D-Galactosamine-Induced Endotoxin Shock

(A) (Panels a and c) Western blots of LPS or PMA-stimulated and unstimulated rested TEPM were probed with antibodies against phospho ERK1, and phospho ERK2 (panel a) or phospho MEK 1 and phospho MEK2 (panel c). Probing the same extracts with antibodies against total ERK1, ERK2, MEK1, and MEK2 revealed that expression was equal in all samples (data not shown). (Panel b) ERK1 and ERK2 immunoprecipitates from the same cell samples were used for in vitro kinase assays. The substrate was GST-Elk1. (Panel d) In vitro kinase assay on ERK5 immunoprecipitates from lysates of unstimulated and LPS or sorbitol-stimulated BMDM. The substrate was MBP.

(B) (Panels a and c) Western blots of LPS-stimulated and unstimulated rested TEPM were probed with antibodies against phospho JNK1 and phospho JNK2 (panel a) or phospho p38 MAPK (panel c). The results show that all these kinases undergo phosphorylation in response to LPS in both *Tpl2* +/+ and the *Tpl2* −/− macrophages. Arrows marked by asterisks indicate cross reacting phosphorylated ERK bands (data not shown).

(Panels b and d) JNK1 (panel b) and JNK2 (data not shown) as well as p38 MAPK (panel d) immunoprecipitates were used for in vitro kinase assays. The substrates were GST-Jun (for JNK) and GST-ATF2 (for p38 MAPK). The p38 MAPK kinase reaction was carried out with cold ATP. Phosphorylation of the substrate was detected by probing a Western blot of the product of the kinase reaction with an antibody against phospho-ATF2 from NEB.

(C1) PD98059-treated and untreated rested TEPM from *Tpl2* +/+ mice were exposed to LPS. TNF-α induction was measured by ELISA in culture supernatants harvested 2 hr later.

(C2) (Panel a) TEPM cultures parallel to the TEPM cultures in (C1) were harvested at 30 min after exposure to LPS. A Western blot of the harvested lysates was probed with the anti-phospho ERK1/phospho ERK2 antibody used in (Aa). The result shows that PD98059 blocks ERK activation. (Panels b and c) Total RNA from the same cells was used in a TNF-α RNase protection assay. The result shows that PD98059 did not inhibit the induction of TNF-α mRNA by LPS. Results similar to the ones shown in (A), (B), and (C1) and (C2) were obtained also with BMDM cultures.

(D1) EMSA of nuclear extracts of LPS-stimulated and unstimulated TEPM from *Tpl2* +/+ and *Tpl2* −/− mice incubated with a ^{32}P-labeled double-stranded oligonucleotide probe corresponding to the NF-κB binding site.

(D2) Ten NF-κB1 −/− mice (8–12 weeks old) and 10 wild-type mice were inoculated with LPS and D-Galactosamine. The bars show percent survival at 5 and 9 hr post inoculation.

The Induction by LPS Depends on Tpl2/ERK-Transduced Signals That Regulate Nucleocytoplasmic mRNA Transport by 3′ARE-Dependent Mechanism

The AU-rich element in the 3′ UTR of the TNF-α mRNA (3′ARE) inhibits TNF-α expression posttranscriptionally (Kontoyiannis et al., 1999). TNF-α-inducing signals target the 3′ARE and relieve its inhibitory effect. To determine whether the Tpl2-transduced LPS signals promote the induction of TNF-α by targeting the 3′ARE, we crossed the *Tpl2* −/− mice to *TNF-α* ΔARE/− mice, which are *TNF-α* heterozygous with one *TNF-α* allele inactivated (−) and a second *TNF-α* allele carrying a deletion of the 3′ARE (ΔARE). After the appropriate crosses were carried out, we obtained mice of the geno-

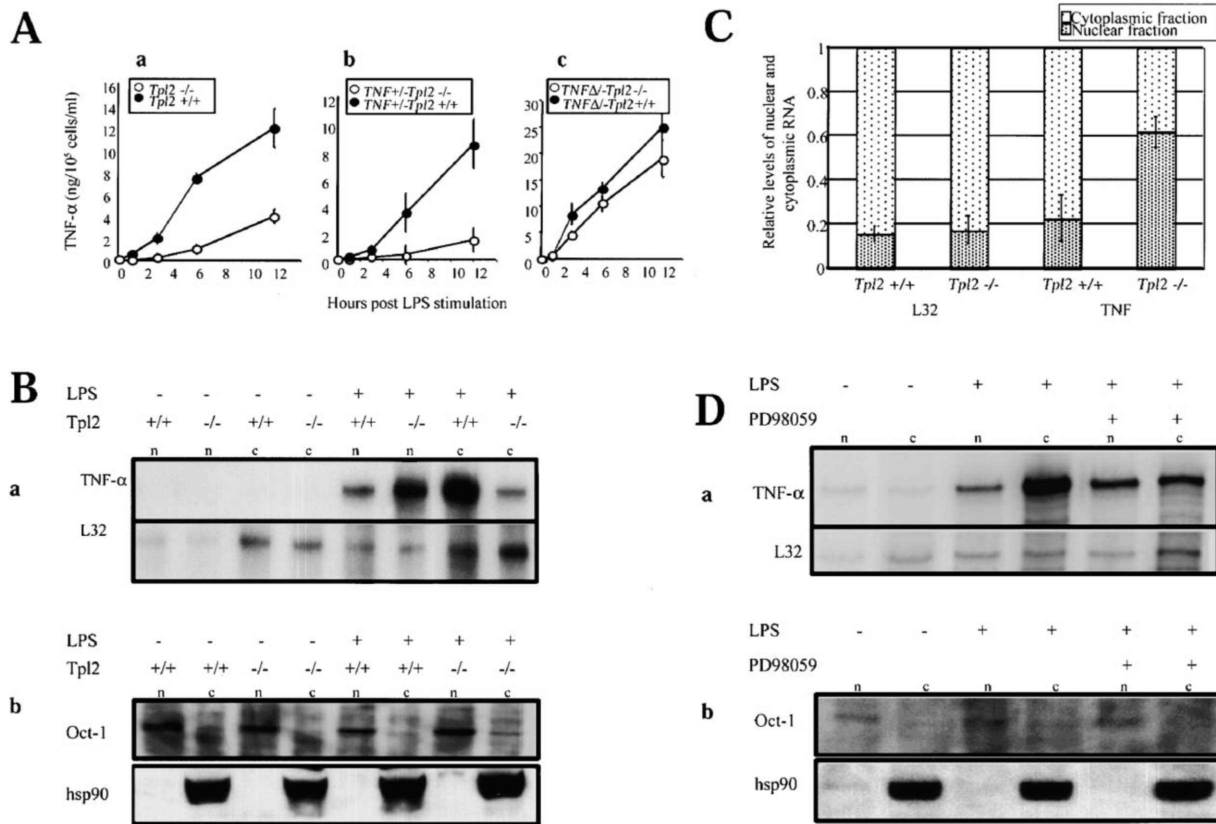


Figure 6. Tpl2 Controls TNF-α Induction by LPS by Transducing ERK Activation Signals That Regulate Nucleocytoplasmic RNA Transport by a 3′ARE-Dependent Mechanism

(A) The effect of Tpl2 on the induction of TNF-α by LPS is influenced by the AU-rich element in the 3′UTR of the TNF-α mRNA. BMDM from *TNF*-α ΔARE/− *Tpl2* −/− and *TNF*-α ΔARE/− *Tpl2* +/+ mice were stimulated with LPS. TNF-α secretion in culture supernatants was measured by ELISA at the indicated time points. BMDM from *Tpl2* +/+ and *Tpl2* −/− as well as from *TNF*-α +/− *Tpl2* −/− and *TNF*-α +/− Tpl2 +/+ mice were used as controls. BMDM from mice of both *TNF*-α ΔARE/−, *Tpl2* +/+ and *TNF*-α ΔARE/− *Tpl2* −/− genotypes express high basal levels of TNF-α and exhibit similar levels of TNF-α induction in response to LPS.

(B) (panel a) Distribution of the TNF-α RNA in the nucleus and cytoplasm. Nuclear and cytoplasmic RNA was extracted from *Tpl2* +/+ and *Tpl2* −/− BMDM before and after stimulation with LPS (1 μg/ml) for 1 hr. Levels of TNF-α and L32 RNA in these fractions were determined by RNase protection. (panel b) Western blots of nuclear and cytoplasmic extracts were probed with antibodies to Oct1 (nuclear protein) and Hsp90 (cytoplasmic protein) to determine the efficiency of nucleocytoplasmic separation.

(C) Relative levels of nuclear and cytoplasmic L32 and TNF-α RNA following LPS stimulation. The experiment in (Ba) was repeated four times with similar results and all bands were quantitated by Phosphorimager. To total RNA (nuclear plus cytoplasmic) for each probe in each experiment was given the numerical value of 1. This allowed the derivation of numerical values for nuclear and cytoplasmic RNA levels that could be compared between experiments. The graph shows the means value ± the standard deviation of the relative levels of nuclear L32 and TNF-α RNAs based on data from all four experiments.

(D) (panel a) Effects of PD98059 treatment on the nucleus and the cytoplasm of *Tpl2* +/+ BMDM before and after LPS stimulation (1 μg/ml). The experimental protocol was the same as in panel Ba. (panel b) Western blots of nuclear and cytoplasmic extracts probed with antibodies to Oct1 and Hsp90 as in (Bb).

types shown in Figure 6A. BMDM from these mice (six mice per group) were stimulated with LPS. Culture supernatants were harvested at the indicated time points and they were analyzed for TNF-α by ELISA. The results showed that the loss of the 3′ARE minimizes the difference in TNF-α induction by LPS between *Tpl2* +/+ and the *Tpl2* −/− cells. Therefore, the Tpl2-transduced ERK1 and ERK2 activation signals responsible for the induction of TNF-α function at least in part in a pathway that targets the 3′ARE of the TNF-α mRNA.

Previous studies on the role of the JNK and p38 MAPK pathways in the induction of TNF-α by LPS suggested that they may enhance translation and perhaps stability of the TNF-α mRNA by targeting its 3′ARE (Kontoyiannis et al., 1999 and references therein). Theoretically, the p38 MAPK pathway may also affect nucleocytoplasmic RNA transport (Kotlyarov et al., 1999) although such a mechanism has never been demonstrated experimentally. Inhibition of any of the three MAPK pathways (JNK, p38 MAPK, and ERK) is sufficient to block induction of TNF-α by LPS (Kontoyiannis et al., 1999; Kotlyarov et al., 1999; and this report). Based on this, we hypothesize that despite the fact that they may all target the 3′ARE of the TNF-α mRNA they may operate by different mechanisms. If this were the case, it would be unlikely for the Tpl2/ERK pathway to regulate the stability of the RNA that may be regulated by JNK and p38 MAPK. Since LPS induces similar TNF-α RNA levels in *Tpl2* +/+

and *Tpl2* −/− macrophages, we conclude that indeed the Tpl2/ERK pathway does not affect RNA stability. To determine whether this pathway affects mRNA transport from the nucleus to the cytoplasm, we carried out subcellular fractionation experiments on 30×10^6 BMDM from *Tpl2* +/+ and *Tpl2* −/− mice before and 1 hr after stimulation with LPS. The relative levels of TNF-α and L32 (control) RNA in the nuclear and cytoplasmic cellular fractions were measured using an RNase protection assay. The results (Figure 6Ba) showed that the ratio of cytoplasmic to nuclear TNF-α mRNA in the *Tpl2* −/− macrophages was low by comparison with the same ratio in *Tpl2* +/+ macrophages. Western blots of the cytoplasmic and nuclear fractions were probed with antibodies to Oct1 (nuclear transcription factor) and Hsp90 (cytoplasmic protein). The results (Figure 6Bb) showed a minimal amount of contamination between compartments. To confirm these data, we repeated the experiment four times and we quantitated the radioactivity of individual bands by PhosphorImager. To combine the quantitative data from all four experiments, we added the TNF-α or L32 nuclear and cytoplasmic RNA levels in the two cell types in each experiment and we assigned to the sum the value of 1. This allowed us to obtain numerical values for the relative levels of nuclear and cytoplasmic RNAs in each experiment. Figure 6Bc shows the mean value ± the standard deviation of the L32 and TNF-α nuclear RNAs in *Tpl2* +/+ and *Tpl2* −/− macrophages based on the results of all four experiments. To confirm that nucleocytoplasmic transport of the TNF-α mRNA is regulated by activated ERK, we repeated the preceding experiment with BMDM derived from *Tpl2* +/+ mice in the presence and absence of the MEK inhibitor PD98059. The results in Figure 6D showed that the separation of nuclear and cytoplasmic fractions was efficient (b) and that PD98059 inhibits the transport. We conclude that nucleocytoplasmic transport of the TNF-α mRNA is indeed regulated by ERK.

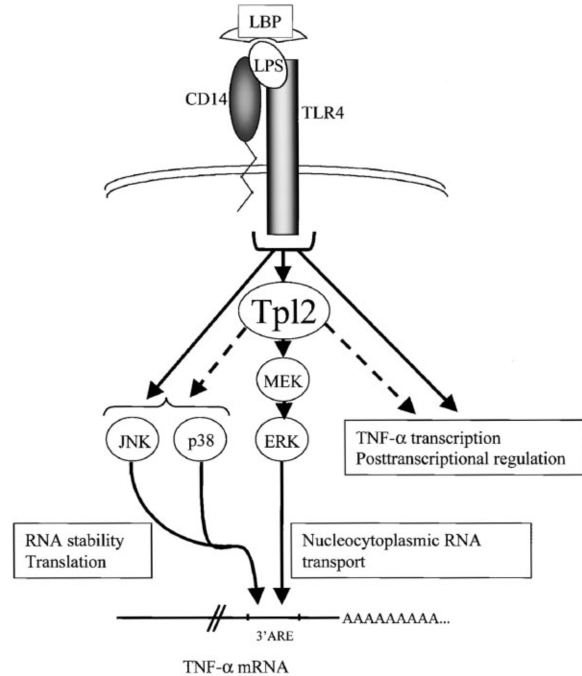


Figure 7. Model for the Posttranscriptional Induction of TNF-α by LPS in Macrophages

The Tpl2/ERK pathway regulates expression of TNF-α by targeting the 3′ARE of the TNF-α mRNA. Moreover, the Tpl2-dependent ERK pathway controls TNF-α expression by regulating the nucleocytoplasmic transport of this mRNA. The JNK, p38 MAPK, and ERK pathways function in concert. The JNK and p38 MAPK pathways appear to regulate translation and perhaps stability of the RNA. Inhibition of any of the three MAPK pathways (ERK, JNK, p38 MAPK) inhibits TNF-α expression. Therefore, despite the fact that they may all target the 3′ARE, their roles are not redundant. Tpl2 may transduce signals that contribute to the activation of all three pathways. However, its role is obligatory only for the activation of the ERK pathway.

Induction of TNF-α by LPS: A Model

Earlier studies had shown that inhibition of the JNK or the p38 MAPK pathways blocks the induction of TNF-α by LPS (Kontoyiannis et al., 1999: Kotlyarov et al., 1999 and references therein). The same studies had suggested that JNK and the p38 MAPK may regulate the induction of TNF-α posttranscriptionally perhaps by targeting the 3′ARE of the TNF-α mRNA. In the present study, we showed that the MAP Kinases ERK1 and ERK2 are also required for TNF-α induction and that their activation by LPS depends on Tpl2. Moreover, we showed that the Tpl2/ERK signals regulate the transport of the TNF-α mRNA from the nucleus to the cytoplasm. Interestingly, these signals regulate the expression of TNF-α by targeting the TNF-α mRNA 3′ ARE, which therefore is likely to be the RNA element responding to the Tpl2/ERK-transduced nuclear export signals. These data combined suggest that all three pathways, ERK, JNK, and p38 MAPK are required for TNF-α induction by LPS and that inactivation of any one of them may block the induction. However, the mechanism by which these pathways regulate this process may differ in that JNK and p38 MAPK may regulate translation and perhaps stability of the mRNA while ERK regulates nucleocytoplasmic mRNA transport (Figure 7). Although all three pathways may target the 3′ARE of the TNF-α mRNA, it is possible that at least some of their effects may be ARE independent.

Discussion

Evidence presented in this report shows that *Tpl2* −/− mice are resistant to LPS/D-Galactosamine-induced endotoxin shock. Their resistance results from a defect in the induction of TNF-α in response to LPS. The defect was observed both in vivo as well as in cultures of unfractionated splenocytes and peritoneal and bone marrow–derived macrophages. Since *Tpl2* −/− splenocytes produce TNF-α in response to signals other than LPS, we conclude that Tpl2 functions at the top of the LPS-induced cascade of TNF-α induction. However, Tpl2 does not function at the level of the LPS receptor. Thus, the expression of CD14, the macrophage high-affinity receptor is normal in *Tpl2* −/− macrophages. Moreover, the *Tpl2* −/− mice secrete nitric oxide in response to LPS and exposure of *Tpl2* −/− macrophages to LPS activates the JNK, p38 MAPK, and NF-κB

pathways. Finally, *Tpl2* −/− and *Tpl2* +/+ splenic B cells proliferate normally following LPS stimulation.

TNF-α induction in response to LPS depends on Tpl2-transduced ERK activation signals that regulate the induction of TNF-α primarily at the posttranscriptional level. Our findings show that the posttranscriptional control of the induction of TNF-α by LPS depends not only on the JNK and p38 MAPK pathways as earlier data indicate (Kontoyiannis et al., 1999 and references therein; Kotlyarov et al., 1999 and references therein), but also on the ERK pathway. One of the targets of the Tpl2/ERK-transduced LPS signals is the 3′ARE of the TNF-α mRNA, which may also be the target of the LPS-activated JNK and p38 MAPK pathways (Kontoyiannis et al., 1999). The mechanism of the functional interaction between these pathways and the 3′ARE has been addressed using genetic strategies or pharmacological inhibitors. These experiments suggested that JNK and p38 MAPK may regulate translation and perhaps stability of the message (Kontoyiannis et al., 1999 and references therein; Kotlyarov et al., 1999). Interestingly, blocking any of the three MAPK pathways (ERK, JNK, or p38 MAPK) is sufficient to inhibit the induction of TNF-α (Kontoyiannis et al., 1999; this report). We conclude, therefore, that despite the fact that they may all target the 3′ARE, they operate by nonredundant mechanisms.

The data presented in this report show that the Tpl2/ERK pathway promotes the nucleocytoplasmic transport of the TNF-α mRNA. Since this study which, using a physiologically relevant system, describes an mRNA nucleocytoplasmic transport mechanism restricted to the transport of a single or a limited number of specific RNAs, this finding defines a novel mechanism of gene regulation and provides the biological framework for new approaches in the study of nucleocytoplasmic RNA trafficking. Future studies will focus on the identification of ERK pathway targets that regulate the nucleocytoplasmic transport of TNF-α mRNA perhaps by binding the 3′ARE. One protein targeted by ERK and p38 MAPK is the serine-threonine kinase Mnk1 (Fukunaga and Hunter, 1997), which has been shown to phosphorylate eIF-4E (Pyronnet et al., 1999). Interestingly, eIF-4E, in addition to its role in translational regulation, also contributes to the nucleocytoplasmic transport of cyclin D mRNA (Rousseau et al., 1996).

Stimulation by LPS leads to the activation of multiple signaling pathways. Several molecules including MyD88, the interleukin-1-associated kinases 1, 2, and M (IRAK-1, -2, -M), and TRAF6 have been shown to contribute to LPS signaling (for review see Anderson, 2000). Interestingly, MyD88 and IRAK-1 knockout mice (Kawai et al., 1999; Swantek et al., 2000), similar to the Tpl2 knockout mice, are also resistant to LPS-induced endotoxin shock. However, the effects of these mutations on LPS signaling appear to be distinct by comparison with the effects of the Tpl2 inactivation. Specifically, inactivation of MyD88 did not prevent but significantly delayed the activation of the ERK, JNK, p38, and NF-κB pathways (Kawai et al., 1999), suggesting that signals transduced by other adaptor molecules that are activated at later time points may also activate these pathways. Therefore, MyD88 may transduce signals targeting Tpl2 but it is unlikely to be the only adaptor molecule carrying out this function. Interestingly, inactivation of MyD88 prevents the activation of IRAK-1 (Kawai et al., 1999) but there is no information regarding the role of MyD88 in the LPS-induced activation of IRAK-2 and IRAK-M. Macrophages from IRAK-1 knockout mice failed to activate the JNK and p38 MAPK pathways and activated the ERK pathway only partially in response to LPS (Swantek et al., 2000). These findings suggest that Tpl2 may function downstream of the interleukin-1 receptor-associated kinases, but that in the absence of IRAK1, it may be a target of IRAK-2 or -M. Since there is no evidence that the TRAF6-deficient mice are defective in ERK activation (Lomaga et al., 1999), it is unlikely that TRAF6 is involved in the regulation of Tpl2.

Earlier studies had suggested that ERK1 and ERK2 may induce TNF-α transcription in human peripheral monocytes (van der Bruggen et al., 1999) and RAW 264.7 (Geppert et al., 1994), a murine macrophage cell line transformed by Abelson MuLV (Raschke et al., 1978). Since we did not observe any effects of the LPS-activated MAPK pathway on TNF-α transcription, we attribute the difference between this and the earlier studies on phenotypic differences between different cell types. We should also add here that the effects of the MAPK pathway on transcription were very weak and that the potential role of the MAPK pathway in the posttranscriptional regulation by TNF-α was not addressed by these studies (Geppert et al., 1994; van der Bruggen et al., 1999).

Previous work had shown that overexpression of Tpl2 activates the ERK1, ERK2, JNK, and p38 MAPK pathways, NFAT and NF-κB (Patriotis et al., 1994; Salmeron et al., 1996; Tsatsanis et al., 1998a., 1998b; Belich et al., 1999; Lin et al., 1999). Here we showed that all these pathways, with the exception of the ERK pathway, can be activated by Tpl2-independent LPS signals in both peritoneal and bone marrow–derived macrophages. Therefore, Tpl2 is obligatory only for the activation of ERK1 and ERK2 by LPS. The apparent lack of involvement of NF-κB in the reported Tpl2 phenotype requires special mention. Thus, our data show that LPS activates both IKKα and IKKβ in peritoneal macrophages from *Tpl2* −/− and *Tpl2* +/+ mice. Moreover, nuclear extracts of LPS-stimulated *Tpl2* −/− and *Tpl2* +/+ peritoneal macrophages exhibit similar levels of NF-κB binding activity. Finally, *NF-κB1* knockout mice (Sha et al., 1995) remain sensitive to LPS/D-Galactosamine-induced endotoxin shock.

Tpl2 is required for the induction of TNF-α by LPS, but not by anti-CD3 plus anti-CD28 or PMA plus ionomycin. This observation is in conflict with observations suggesting that Tpl2 contributes to the induction of TNF-α in response to anti-CD3 stimulation in Jurkat cells (Ballester et al., 1998). This is reminiscent of an earlier observation showing that even though Tpl2 plays a critical role in the induction of IL-2 in Jurkat cells (Tsatsanis et al., 1998a), its role in the induction of IL-2 in primary splenocytes in response to anti-CD3 plus anti-CD28 is dispensable (this report). These differences are likely to be the result of the improper activation or inactivation of complementing signaling pathways in tumor cells, and they point out that observations made on tumor cells lines, although useful, should be viewed with caution.

The data presented in this report clearly show that

Tpl2 is required for the induction of endotoxin shock in LPS-inoculated mice because it transduces LPS signals that control the expression of TNF-α. Since *Tpl2* −/− mice are at least as susceptible as the *Tpl2* +/+ mice to toxic shock induced by TNF-α and D-Galactosamine, we conclude that Tpl2 is not required for the transduction of TNF-α signals responsible for the pathophysiology of the endotoxin shock syndrome. These results, however, do not exclude the possibility that Tpl2 may contribute to the transduction of TNF-α signals. Preliminary data indeed suport the involvement of Tpl2 in the transduction of TNF-α-generated signals (Kontoyiannis et al., unpublished results; Patriotis et al., unpublished results). Such signals, if confirmed, will not have a major role in the pathophysiology of endotoxin shock, but they may contribute to other TNF-α-dependent inflammatory syndromes.

The findings presented in this manuscript provide definitive information regarding the physiological signaling role of the *Tpl2* protooncogene. In addition, they show that the inactivation of *Tpl2* is not only well tolerated in vivo, but also provides protection from LPS-induced endotoxin shock. These observations therefore identify Tpl2 as an excellent target for the development of drugs against inflammation and the septic shock syndrome.

Experimental Procedures

Mice

Homologous recombination between the targeting construct (Figure 1A) electroporated into the 129/SV mouse-derived ES cell line CJ7 and the endogenous *Tpl2* gene replaced the Tpl2 sequences between exons 3 and 5 with the G418 resistance cassette. Several of the resulting mutant ES cell clones were injected into blastocysts derived from C57Bl/6 mice. The resulting chimeras were mated to C57Bl/6 mice and the *Tpl2* +/− mice, derived from this cross, were backcrossed to C57Bl/6 for nine generations. Mice utilized in the experiments presented here were obtained by brother–sister mating of *Tpl2* −/− and *Tpl2* +/+ animals.

Mice were genotyped for their *Tpl2* status by hybridizing Southern blots of SphI–digested DNA with probes 1 and 2. Expression of Tpl2 was measured in spleen by Northern blotting using polyA+ RNA.

TNF-α ΔARE/− mice are double heterozygotes for two TNF-α mutations: one that deleted the ARE element in the 3′ UTR of the *TNF-α* mRNA (ΔARE) (Kontoyiannis et al., 1999) and a second one that inactivated the other *TNF-α* allele (−) (Kontoyiannis et al., 1999). These mice were crossed to ninth generation *Tpl2* −/− mice to generate *TNF-α* ΔARE/+, *Tpl2* +/−, and *TNF-α* +/−, *Tpl2* +/− F1 animals. Mice of these two genotypes were crossed to each other to generate *TNF-α* ΔARE/−, Tpl2 +/+, and *TNF-α* ΔARE/−, Tpl2 −/− double mutant mice.

NF-κB1 −/− mice (Sha et al., 1995) were purchased from the Jackson Labs.

Histology, cell population profiles in hematopoietic organs, ELISA assays for antibody and cytokine levels in animals, and in culture and cell proliferation assays were carried out using standard protocols.

LPS/D-Galactosamine-Induced Endotoxin Shock/ TNF-α Toxicity

8- to 12-week-old *Tpl2* −/−, *NF-κB1* −/−, and control mice of both sexes were inoculated IP with 1 mg/g body weight D-Galactosamine (Sigma) (250 mg/ml in PBS) and with 0.1, 1, or 10 μg lipopolysaccharide (LPS) derived from *Salmonella enteritidis* (Sigma) (10, 100, or 1000 μg/ml solution in PBS). The syndrome arising from this treatment culminated in death at 6 to 9 hr. In separate identical experiments, the mice were bled at 30 min and 1 hr after treatment and the concentration of TNF-α was measured in the serum by ELISA. The concentration of IL1-β was measured also by ELISA at 2 hr after treatment.

8- to 12-week-old mice of both sexes were also used for TNF-α toxicity studies. These mice were inoculated IP with 1 mg/g body weight D-Galactosamine. 1 hr later, they were inoculated IV with 35 ng/g body weight TNF-α (Endogen, Woburn, MA) and they were placed under observation.

Cell Culture

Single cell suspensions of splenocytes were cultured using standard protocols.

To culture thioglycollate-elicited peritoneal macrophages (TEPM), mice were injected IP with 1.5 ml thioglycollate broth (Sigma). Four days later, the mice were sacrificed and their peritoneal cavities were washed three times with 5 ml of cold Hanks balanced salt solution (HBSS) (Gibco). Cell pellets were washed once with DMEM supplemented with 10% FBS and they were cultured at the concentration of 10^6 cells/ml. 2 hr later the dishes were washed with 10 ml of medium to remove nonadherent cells. At least 95% of the remaining adherent cells were macrophages (data not shown). Cells were stimulated with LPS (1 μg/ml) or PMA (100 nM). Prior to stimulation, TEPM were cultured overnight in DMEM supplemented with only 0.5% FBS.

Bone marrow–derived macrophages (BMDM) were isolated and cultured as described (Warren and Vogel, 1985). In short, the bone marrow was flushed from femurs of wild-type and mutant mice with 5 ml of DMEM supplemented with 10% FBS. Cell pellets were resuspended in ACK hypotonic buffer, to remove red blood cells, and were subsequently washed with DMEM with 10% FBS and cultured at the concentration of 10^7 cells/ml in DMEM supplemented with 20% FBS and 30% L929 cell conditioned media (CSF-1 source) in bacteriological Petri dishes. Six days later, adherent macrophages were trypsinized, counted, and replated to be used experimentally. Cells were treated with LPS (1 μg/ml), PMA (100 nM) or sorbitol (0.5 M). Prior to stimulation, BMDM were cultured overnight in DMEM supplemented with only 0.5% FBS.

The reconstitution of *Tpl2* −/− BMDM was carried out by infecting these macrophages with a high titer virus derived from a pMSCV retrovirus construct that expresses Tpl2 wt and green fluorescent protein (GFP) from two independent promoters (Patriotis, unpublished results). Two days after the infection, the cells were stimulated with LPS in the presence of monensin. TNF-α expression was scored 4 hr later in green fluoresceing cells (expressing Tpl2) and nonfluorescing cells (not expressing Tpl2) by flow cytometry.

ELISA

To measure TNF-α in serum and in culture supernatants of splenocytes or macrophages or in sera, we used an ELISA kit from Pharmingen (OptEIA TNF-α kit) and we followed the manufacturer's protocol. IL-1β, IL-2, IL-4, and IFNγ were measured using similar ELISA kits purchased from Biosource (Camarillo, CA).

Measurement of Intracellular TNF-α in TEPM and BMDM

Intracellular TNF-α was measured in TEPM and BMDM stimulated with 1 μg/ml LPS from *Salmonella enteritidis* for 6 hr. To inhibit transport to the plasma membrane and subsequent cleavage and release of TNF-α, cells were treated with 2 μM of monensin (Golgi-Stop, Pharmingen). Cells were scraped off the Petri dish and washed in PBS supplemented with 5% calf serum. Subsequently, they were permeabilized by resuspending them in PBS containing 5% calf serum and 0.1% (w/v) saponin (Sigma). Intracellular TNF-α was stained by incubating the permeabilized cells with anti-TNF-α FITC-labeled antibody from Pharmingen (A6088) (1.2 μg/ml final concentration) for 30 min on ice. Following washing with PBS containing 5% calf serum, stained cells were identified by flow cytometry.

Northern Blotting and RNase Protection Assays

RNA was extracted from spleens of mice treated with LPS and D-Galactosamine or from LPS-stimulated cultured TEPM and BMDM using the Qiaquick kit from Qiagen (Qiagen, Valencia, CA) or using the method of Chomczynski (Chomczynski, 1993). In short, cells were lysed in buffer containing guanindinium thiocyanate and the RNA was extracted from the lysates with acid phenol. In some of the experiments, BMDM were fractionated into nuclear and cytoplasmic fractions using the NePer kit (Pierce, Rockford, IL) and

following the manufacturer's protocol (Kaboord et al., 2000). Total RNA (12 μg) was subjected to electrophoresis in denaturing formaldehyde gels. Following this, the RNA was transferred to nylon membranes (MagnaGraph from MSI/Osmonics) and it was hybridized to cDNA probes for TNF-α and mouse actin (loading control). RNase protection was carried out with 2 μg of total RNA from spleen cells and peritoneal or bone marrow–derived macrophages using an RNase protection assay kit (Pharmingen, San Diego, CA) and following the manufacturer's instructions. The RNA/RNA hybrid complexes remaining after digestion of the nonhybridizing RNA with RNase A and RNase T1 were separated in 6% denaturing sequencing gels. Individual bands in both the Northern blots and the RNase protection experiments were quantitated using a Molecular Dynamics PhosphorImager. To determine the efficiency of nucleocytoplasmic separation, we probed Western blots of the nuclear and cytoplasmic fractions with antibodies from Santa Cruz directed against Oct1 nuclear transcription factor and against the cytoplasmic protein Hsp90 (Kaboord et al., 2000).

Western Blotting

Cells were lysed in SDS sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromphenol blue) and the lysates were subjected to ultrasonication and 5 min boiling. Protein concentration was measured in 1:200 diluted lysates using the Bradford assay (BioRad). Following SDS-polyacrylamide gel electrophoresis of the extracts (50 μg protein per lane), proteins were transferred to PVDF nylon membranes. Membranes were blocked in TBS (Tris-buffered saline) containing 0.1% Tween-20 and 5% milk. Primary antibodies were added in 5% milk (for anti-phospho-ERK1/ERK2 [Thr202/Tyr204] and anti-ERK1/ERK2) or in 5% BSA (for anti-phospho-MEK1/MEK2 [Ser217/221], anti-phospho-p38 MAPK [Thr180/Tyr182], anti-phospho-JNK1/2 [Thr183/Tyr185], anti-MEK1/2, anti-p38 MAPK, and anti-JNK1/2) for a minimum 16 hr at 4°C. All antibodies were rabbit polyclonals and they were purchased form New England Biolabs (Beverly, MA). Excess antibodies were washed at room temperature three times for 5 min with TBS containing 0.1% Tween-20. Washed membranes were incubated at room temperature for 1 hr with goat anti-rabbit antiserum conjugated with horseradish peroxidase. Excess antiserum was washed three times with TBS-Tween-20 at room temperature. Antigen-antibody complexes were detected by chemiluminescence.

In Vitro Kinase Assays

Cells were lysed in Triton-X-100 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 mM PMSF). ERK1/ERK2, JNK1, JNK2, and ERK5/BMK1 were immunoprecipitated from 100–500 μg of cellular extracts using antibodies from Santa Cruz (ERK1/2, JNK1, ERK5) or Upstate Biotechnology (JNK2) and 20 μl of protein A-agarose beads (Gibco). After overnight incubation, the beads were washed twice in lysis buffer and twice in kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). The washed immunoprecipitates were incubated at 25°C for 30 min with 20 μM ATP, 5 μCi [γ-^{32}P]-ATP [3000 mCi/mmol] and 1 μg of purified substrates in kinase buffer. The substrates were GST-Elk1 fusion for ERK1/ERK2 kinase assays), GST-Jun fusion (for JNK1 and JNK2 kinase assays), and myelin basic protein (MBP) for ERK5/BMK1. The p38 MAPK assay was carried out with an NEB kit. Phosphorylation of the GST-ATF2 substrate was detected by Western blotting with an anti-phospho ATF2 (Thr71) specific antibody (NEB).

Electrophoretic Mobility Shift Assay

Nuclear extracts of TEPM treated with LPS for 60 min were prepared using the NePer kit (Pierce, Rockford, IL). Cells harvested by scraping in PBS were pelleted and lysed in 200 μl of an NP-40 low salt lysis buffer. Intact nuclei were pelleted by centrifugation at 12,000 × g and they were lysed in 50 μl of a high-salt buffer. The protein in the final lysates was quantitated using the Bradford assay (BioRad). One microgram of nuclear extracts was incubated with 10^5 cpm of a ^{32}P-labeled double-stranded DNA probe representing the NF-κB binding site (5′-ACAAGGGACTTTCCGCTGGGGACTTTCCAGGG-3′). Incubation was carried out for 30 min on ice in a binding buffer containing 20 mM HEPES (pH 7.5), 0.5 mM EDTA, 5 mM $MgCl_2$, 50 μg/ml bovine serum albumin, 0.05% NP-40, 60 mM KCl, 10 mM DTT, 10% glycerol, and 1.5 μg of poly(dI/dC). Bound probe was separated from free probe by electrophoresis in a 6% nondenaturing polyacrylamide gel. Electrophoresis was carried out at 5 V/cm for approximately 1.5 hr. The double-stranded probe was generated by annealing two complementary oligonucletides and by filling the ends using Klenow polymerase and radioactive nucleotides.

Acknowledgments

The authors wish to thank Dr. K. Hayakawa for help in measuring the antibody response to the T cell-dependent antigen KLH, Dr. G. Rall for measuring T cell cytotoxic responses to LCMV, and Dr. A. Klein-Szanto for help with the interpretation of histology. The authors also wish to thank Drs. E. Alnemri, S. Bear, T. Manser, and Y. Sykulev for critical review of the manuscript and Dr. T. O. Chan for helpful discussions. The work was supported by Public Health Service grant R01 CA38047 (to P. N. T.), by the National Cancer Institute (N. G. C.) and by the Hellenic General Secretariat of Research and Technology and EC grants QLG1-1999-00202 and QLK6-1999-02203 (to G. K.). C. P. was a special fellow of the Leukemia Society of America Inc. (now The Leukemia and Lymphoma Society).

Received April 7, 2000; revised November 10, 2000.

References

Anderson, K.V. (2000). Toll signaling pathways in the innate immune response. Curr. Opin. Immunol. *12*, 13–19.

Ballester, A., Velasco, A., Tobena, R., and Alemany, S. (1998). Cot kinase activates tumor necrosis factor-alpha gene expression in a cyclosporin A-resistant manner. J. Biol. Chem. *273*, 14099–14106.

Belich, M.P., Salmeron, A., Johnston, L.H., and Ley, S.C. (1999). TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105. Nature *397*, 363–368.

Ceci, J.D., Patriotis, C.P., Tsatsanis, C., Makris, A.M., Kovatch, R., Swing, D.A., Jenkins, N.A., Tsichlis, P.N., and Copeland, N.G. (1997). Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation. Genes Dev. *11*, 688–700.

Chiariello, M., Marinissen, M.J., and Gutkind, J.S. (2000). Multiple mitogen-activated protein kinase signaling pathways connect the Cot oncoprotein to the c-jun promoter and to cellular transformation. Mol. Cell. Biol. *20*, 1747–1758.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques *15*, 532–537.

Dinarello, C.A., Gelfand, J.A., and Wolff, S.M. (1993). Anticytokine strategies in the treatment of the systemic inflammatory response syndrome. JAMA *269*, 1829–1835.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., and Saltiel, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. USA *92*, 7686–7689.

Erny, K.M., Peli, J., Lambert, J.F., Muller, V., and Diggelmann, H. (1996). Involvement of the Tpl-2/cot oncogene in MMTV tumorigenesis. Oncogene *13*, 2015–2020.

Fink, M.P., and Heard, S.O. (1990). Laboratory models of sepsis and septic shock. J. Surg. Res. *49*, 186–196.

Fukunaga, R., and Hunter, T. (1997). MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J. *16*, 1921–1933.

Galanos, C., Freudenberg, M.A., and Reutter, W. (1979). Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA *76*, 5939–5943.

Geppert, T.D., Whitehurst, C.E., Thompson, P., and Beutler, B. (1994). Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. Mol. Med. *1*, 93–103.

Kaboord, B., Pasley, P., Koelbl, J., Ridnour, H., Devitt, D., Chu, R.,

and Ignacio, R. (2000). NE-PER nuclear and cytoplasmic extraction reagents. Previews—Pierce Chemicals *3*, 4–5.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity *11*, 115–122.

Kontoyiannis, D., Pasparakis, M., Pizarro, T.T., Cominelli, F., and Kollias, G. (1999). Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU- rich elements: implications for joint and gut-associated immunopathologies. Immunity *10*, 387–398.

Kotlyarov, A., Neininger, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H.D., and Gaestel, M. (1999). MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. Nat. Cell Biol. *1*, 94–97.

Leist, M., Gantner, F., Bohlinger, I., Germann, P.G., Tiegs, G., and Wendel, A. (1994). Murine hepatocyte apoptosis induced in vitro and in vivo by TNF-alpha requires transcriptional arrest. J. Immunol. *153*, 1778–1788.

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., and Salfeld, J. (1995). Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell *80*, 401–411.

Lin, X., Cunningham, E.T., Jr., Mu, Y., Geleziunas, R., and Greene, W.C. (1999). The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-kappaB acting through the NF-kappaB-inducing kinase and IkappaB kinases. Immunity *10*, 271–280.

Lomaga, M.A., Yeh, W.C., Sarosi, I., Duncan, G.S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., et al. (1999). TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev. *13*, 1015–1024.

Medzhitov, R., and Janeway, C.A., Jr. (1997a). Innate immunity: the virtues of a nonclonal system of recognition. Cell *91*, 295–298.

Medzhitov, R., and Janeway, C.A., Jr. (1997b). Innate immunity: impact on the adaptive immune response. Curr. Opin. Immunol. *9*, 4–9.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A, .Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394–397.

Parillo, J.E. (1993). Pathogenic mechanisms of septic shock. N. Engl. J. Med. *328*, 1471–1477.

Pasparakis, M., Alexopoulou, L., Episkopou, V., and Kollias, G. (1996). Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J. Exp. Med. *184*, 1397–1411.

Patriotis, C., Makris, A., Bear, S.E., and Tsichlis, P.N. (1993). Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. Proc. Natl. Acad. Sci. USA *90*, 2251–2255.

Patriotis, C., Makris, A., Chernoff, J., and Tsichlis, P.N. (1994). Tpl-2 acts in concert with Ras and Raf-1 to activate mitogen-activated protein kinase. Proc. Natl. Acad. Sci. USA *91*, 9755–9759.

Pfeffer, K., Matsuyama, T., Kundig, T.M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Kronke, M., and Mak, T.W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell *73*, 457–467.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Pyronnet, S., Imataka, H., gingras, A.C., Fukunaga, R., Hunter, T., and Sonenberg, N. (1999). Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E. EMBO J. *18*, 270–279.

Raabe, T., Bukrinsky, M., and Currie, R.A. (1998). Relative contribution of transcription and translation to the induction of tumor necrosis factor-alpha by lipopolysaccharide. J. Biol. Chem. *273*, 974–980.

Raschke, W.C., Baird, S., Ralph, P., and Nakoinz, I. (1978). Functional macrophage cell lines transformed by Abelson leukemia virus. Cell *15*, 261–267.

Rousseau, D., Kaspar, R., Rosenwald, I., Gehrke, L., and Sonenberg, N. (1996). Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E. Proc. Natl. Acad. Sci. USA *93*, 1065–1070.

Salmeron, A., Ahmad, T.B., Carlile, G.W., Pappin, D., Narsimhan, R.P., and Ley, S.C. (1996). Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. EMBO J. *15*, 817–826.

Schletter, J., Heine, H., Ulmer, A.J., and Rietschel, E.T. (1995). Molecular mechanisms of endotoxin activity. Arch. Microbiol. *164*, 383–389.

Sha, W.C., Liou, H.C., Tuomanen, E.I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell *80*, 321–330.

Swantek, J.L., Tsen, M.F., Cobb, M.H., and Thomnas, J.A. (2000). IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. J. Immunol. *164*, 4301–4306.

Tsatsanis, C., Patriotis, C., Bear, S.E., and Tsichlis, P.N. (1998a). The Tpl-2 protooncoprotein activates the nuclear factor of activated T cells and induces interleukin 2 expression in T cell lines. Proc. Natl. Acad. Sci. USA *95*, 3827–3832.

Tsatsanis, C., Patriotis, C., and Tsichlis, P.N. (1998b). Tpl-2 induces IL-2 expression in T-cell lines by triggering multiple signaling pathways that activate NFAT and NF-kappaB. Oncogene *17*, 2609–2618.

Uchida, N., Smilowitz, H., Ledger, P.W., and Tanzer, M.L. (1980). Kinetic studies of the intracellular transport of procollagen and fibronectin in human fibroblasts. Effects of the monovalent ionophore, monensin. J. Biol. Chem. *255*, 8638–8644.

Ulevitch, R.J., and Tobias, P.S. (1995). Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu. Rev. Immunol. *13*, 437–457.

van der Bruggen, T., Nijenhuis, S., van Raaij, E., Verhoef, J., and van Asbeck, B.S. (1999). Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the raf-1/MEK1–MEK2/ERK1–ERK2 pathway. Infect. Immun. *67*, 3824–3829.

Wang, S., Miura, M., Jung, Y.K., Zhu, H., Li, E., and Yuan, J. (1998). Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell *92*, 501–509.

Warren, M.K., and Vogel, S.N. (1985). Bone marrow-derived macrophages: development and regulation of differentiation markers by colony-stimulating factor and interferons. J. Immunol. *134*, 982–989.