THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Anthrax Lethal Toxin Inhibits Activation of IFN-Regulatory Factor 3 by Lipopolysaccharide[1]

Oanh Dang, Lorena Navarro, Keith Anderson, and Michael David[2]

***IFN-regulatory factor 3 (IRF3) is known to participate in the transcriptional induction of chemokines and cytokines, including IFNs, as a result of viral or bacterial infection. In this study, we demonstrate that the LPS-mediated activation of IRF3 and subsequent induction of chemokine genes or IRF3-responsive reporter constructs are inhibited after exposure of human or murine macrophages to the* Bacillus anthracis *toxin lethal factor. The inhibitory effect is caused by interference with the activation of the stress-activated protein kinase, p38, due to a proteolytic cleavage of mitogen-activated protein kinase kinase 6, and can be overcome by the ectopic expression of a cleavage-resistant mutant of mitogen-activated protein kinase kinase 6 or a constitutively active IRF3. The lethal factor-mediated inhibition of IRF3 activation and subsequent cytokine production through bacterial membrane components offers* Bacillus anthracis *an efficient mechanism to evade the innate immune response. The Journal of Immunology,* 2004, 172: 747–751.**

Vertebrates and invertebrates respond to viral infection or bacterial invasion by activation of a defense mechanism that is part of the innate immune response. Innate immune recognition of bacterial infection is mediated by a system of germline-encoded receptors (Toll-like receptors; TLRs)[3] that recognize conserved molecular patterns associated with microbial pathogens such as bacterial cell wall LPS (1). These receptors, which are coupled to downstream signaling cascades that mediate the induction of immune response genes, represent the most ancient host defense system found in mammals, insects, and plants. In mammals, it is primarily monocytes and macrophages that respond to LPS, releasing cytokines and chemokines that provoke an inflammatory response. In addition to the extensively studied NFκB pathway (2, 3), TLRs also stimulate signaling cascades that lead to the activation of the stress-activated protein kinases (SAPKs) c-Jun N-terminal kinase JNK and p38 via members of the mitogen-activated protein (MAP) kinase kinase (MKK) family (4–7). The ubiquitously expressed IFN-regulatory factor 3 (IRF3) is an important cellular response factor to viral infection (8–11), mediating the transcriptional induction of IFNs as well as other chemokines and cytokines. Similarly, IRF3 activation is involved in mediating adequate transcriptional responses toward bacterial components (12).

LPS-mediated transcriptional activation via IRF3 requires the p38 SAPK (12), and involves phosphorylation of serine residues located in two clusters at the C terminus of IRF3 protein (9, 10, 13). Upon its subsequent nuclear translocation, IRF3 binds to IFN-stimulated response element (ISRE)-like enhancers found in a wide variety of genes, where it is either sufficient to induce transcription on its own or in cooperation with other transcription factors (11, 14).

Lethal toxin (LT), produced by *Bacillus anthracis*, is composed of the subunits protective Ag (PA) and lethal factor (LF) (15). PA is a pore-forming protein secreted by *B. anthracis* as a precursor that, upon binding to a specific cell surface receptor and proteolytic activation, mediates entry of LF into the cell. LF is a Zn^{2+}-dependent metalloprotease, which cleaves short N-terminal fragments of several MKK isoforms (16, 17). This cleavage of MKKs does not appear to correlate with the cytotoxic effects observed in certain macrophage and macrophage cell lines but rather is accompanied by a decrease in cytokine and chemokine production (18). However, no information is available on the transcription factors that are targeted in their activation process by *B. anthracis* toxins.

Materials and Methods

Cells

Human U373 astrocytoma cells (U-373 MG) stably transfected with human CD14 were generously provided by Dr. P. Tobias (19). Peritoneal macrophages were collected 5 days after i.p. administration of thioglycolate.

Reagents

LPS was purchased from Sigma-Aldrich (St. Louis, MO). IRF3 and STAT1 antisera have been previously described (12). Abs against IκB and P-p38 were from Cell Signaling (Beverly, MA), p38 mAb was from BD Transduction Laboratories (Lexington, KY), and p65 and MKK3 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). LF and PA were from List Biological Laboratories

Division of Biological Sciences and University of California San Diego Cancer Center, University of California San Diego, La Jolla, CA 92093

Received for publication September 22, 2003. Accepted for publication November 14, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant AI47182 (to M.D.).

[2] Address correspondence and reprint requests to Dr. Michael David, Department of Biology, University of California, San Diego, Bonner Hall 3138, 9500 Gilman Drive, La Jolla, CA 92093-0322. E-mail address: midavid@ucsd.edu

[3] Abbreviations used in this paper: TLR, Toll-like receptor; SAPK, stress-activated protein kinase; MAP, mitogen-activated protein; MKK, MAP kinase kinase; IRF3, IFN-regulatory factor 3; ISRE, IFN-stimulated response element; LT, lethal toxin; PA, protective Ag; LF, lethal factor; wt, wild type; ISG, IFN-stimulated gene.

0022-1767/04/$02.00

(Campbell, CA). Mammalian expression vector encoding IRF3-5D was a gift from Dr. J. Hiscott.

MKK cleavage-resistant mutants

MKK3 and MKK6 expression plasmids were a gift from Dr. J. Han (20). MKK mutants were generated using the Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

Treatments and extracts

Cells were exposed to 200 ng/ml PA and 100 ng/ml LF for 8 h before stimulation with LPS (1 μg/ml for 6 h) or IFN-β (1000 U/ml for 2 h) unless indicated otherwise. For whole cell lysates, cells were lysed with lysis buffer (1 ml) containing 20 mM HEPES (pH 7.4), 1% TX-100 (Sigma-Aldrich), 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM $NaVO_4$, and 1 mM PMSF. Lysates were centrifuged, and protein concentration was determined by the method of Lowry et al.

Western blotting

Cell lysates were resolved by SDS-PAGE, and blots were immunoblotted with the indicated Abs and developed with HRP-conjugated secondary Abs and ECL.

EMSA

EMSAs were performed using ^{32}P-labeled probes corresponding to the ISRE of the *ISG54* gene or the NFκB binding site. Lysates were incubated with poly(deoxyinosinate-deoxycytidylate) and labeled probes in binding buffer (40 mM KCl, 20 mM HEPES (pH 7.0), 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 4% Ficoll, 0.2% Nonidet P-40), and electrophoresis was performed on 6% Tris-buffered EDTA-PAGE. For supershift experiments, extracts were incubated with the specified antiserum for 1 h at 4°C, and the samples resolved on 4.5% Tris-buffered EDTA-PAGE.

RNase protection assay

Total RNA was isolated using TRIzol (Life Technologies, Gaithersburg, MS). ^{32}P-Labeled antisense riboprobes for IFN-stimulated gene (ISG) 54 and GAPDH were generated from the linearized plasmid using T7 or SP6 RNA polymerase. Labeled riboprobe and 10 μg of RNA were incubated in hybridization buffer at 56°C before digestion with T1 RNase for 1 h at 37°C. Protected fragments were solubilized in RNA loading buffer and subjected to electrophoresis on a 4.5% polyacrylamide-urea gel.

RT-PCR

cDNA was prepared from total RNA using the SuperScript First-Strand Synthesis System (InVitrogen Scientific, Santa Ana, CA). cDNAs for RANTES and β-actin were amplified in the same reaction using a *Taq*PCR Core Kit (Qiagen, Valencia, CA).

Results and Discussion

Our previous work demonstrated a role for p38 and IRF3 in the LPS- mediated induction of ISRE-containing genes (12) as exemplified by the complete inhibition of *ISG54* expression in response to LPS by the p38-specific inhibitor SB202190 (Fig. 1*A, lane 3*). To address the concern that SB202190 might also affect the related JNK kinase, we verified the specificity of its inhibitory effects by analyzing the JNK-mediated phosphorylation of c-Jun in response to LPS. As anticipated, SB202190 did not affect LPS-induced c-Jun phosphorylation at the concentration required to inhibit ISG54 induction.

To further characterize these requirements for the LPS-mediated induction of ISRE-controlled genes, we isolated peritoneal macrophages from IRF3$^{-/-}$ or JNK1$^{-/-}$ mice and analyzed their transcriptional responses toward LPS. As shown in Fig. 1*C,* the absence of JNK1 is of no consequence to LPS-induced RANTES expression (*lanes 5* and *6*), whereas the lack of IRF3 expression prohibits the induction of this chemokine (*lanes 3* and *4*). Similar results were seen when ISG54 mRNA was evaluated (data not shown). These findings not only demonstrate the crucial role of IRF3 in the LPS-mediated induction of ISGs but also support a role for p38, but not JNK, in the activation process.

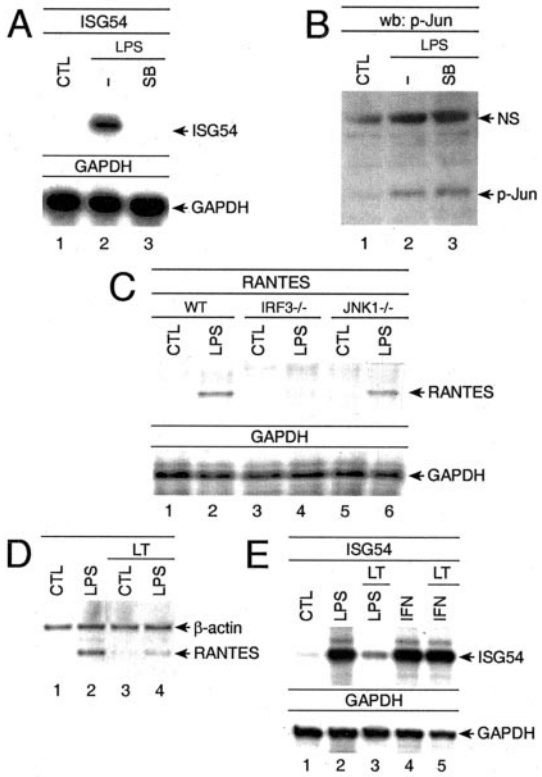

FIGURE 1. Effects of LT on LPS induction of ISRE-controlled genes. *A,* U373 cell were stimulated with LPS in the absence or presence of 10 μg/ml SB202190 (SB) for 6 h. ISG54 mRNA levels were determined by RNase protection assay. *B,* Same as *A,* except stimulation were conducted for 30 min, and total cell lysate was probed for p-Jun. wb, Western blot. *C,* Peritoneal macrophages derived from wt, IRF3$^{-/-}$, and JNK1$^{-/-}$ mice were treated with LPS, and RANTES and actin mRNA levels were determined by RT-PCR. *D* and *E,* U373 cells were exposed to LT for 8 h before stimulation with LPS or IFN-β, respectively. Total RNA was isolated, and RANTES (*D*) and ISG54 (*E*) mRNA levels were determined by RT-PCR and RNase protection assays, respectively.

The presence of *B. anthracis* LT affects the expression of numerous cytokine and chemokine genes (18). We therefore investigated the effects of LT on the LPS-mediated induction of ISRE-controlled genes. U373 cell were exposed to LT for 8 h before stimulation with LPS. Indeed, LPS treatment triggered a significant increase in RANTES mRNA levels (Fig. 1*D, lane 2*) which was almost completely inhibited by LT (Fig. 1*D, lane 4*). Transcriptional induction of the *ISG54* and *RANTES* genes requires binding of IRF3 to the ISRE in the respective promoters; in addition, transcription of *RANTES* is further enhanced through the activation of NFκB (12, 14). To test whether LT was acting on IRF3 or NFκB, we analyzed the effect of LT on the transcriptional induction of *ISG54*, which is regulated exclusively via an ISRE responsive to either LPS-activated IRF3 or the IFN-β-activated STAT1/STAT2 heterodimeric complex, ISGF3 (12). As we reported previously, LPS induces *ISG54* in a protein synthesis-independent manner (Fig. 1*E, lane 2*). However, pretreatment with LT abrogated this LPS-induced ISG54 expression (Fig. 1*E, lane 3*). Importantly, the IFN-β-mediated induction of ISG54 was unaffected by LT (Fig. 1*E, lanes 4* and *5*). An identical effect of LT on LPS-mediated ISG54 induction was observed when primary human peripheral blood monocytes were used in the experiments (data not shown).

Several members of the MKK family are targets for the proteolytic action of LF (16, 17). Because our findings indicated a role for p38 rather than JNK in the activation of ISGs via IRF3, we focused on MKK3 and MKK6, which act as activators of p38. In U373 cells, sublethal doses of LT, but not PA or LF alone, caused a proteolytic degradation of MKK3 (Fig. 2*A*) and MKK6 (Fig. 2*B*), independent of LPS (Fig. 2, *A* and *B, lanes 2* and *6*). Stimulation of endotoxin-responsive cells with LPS causes a rapid and sustained phosphorylation of p38 (Fig. 2*C, lanes 3* and *5*). However, as an apparent consequence of the LT-mediated degradation of MKK3 and MKK6, this LPS-induced p38 activation was completely abrogated in cells exposed to LT (Fig. 2*C, lanes 4* and *6*). LPS stimulation of cells activates NFκB through the proteolytic degradation of IκB (3). Intriguingly, LT did not prevent LPS-induced IκB degradation (Fig. 2*D, lanes 4* and *6*).

We hypothesized that LPS-mediated activation of IRF3 might be impaired in LT-pretreated cells. To specifically test for IRF3 activation, we analyzed its ability to bind an ISRE probe in response to LPS stimulation using in vitro DNA binding assays. Extracts derived from LPS treated cells clearly displayed IRF3 binding to the ISRE (Fig. 3*A, lanes 3* and *6*), as further verified by supershifts with IRF3 and control antisera (Fig. 3*A, lanes 7* and *8*). However, no IRF3/ISRE interaction was observed in cells exposed to LT before stimulation with LPS (Fig. 3*A, lane 4*). Similar results were seen when lipoteichoic acid derived from Gram-positive bacteria was used for stimulation (data not shown). Importantly, the LPS-induced activation of NFκB is not affected by LT as evidenced by intact NFκB DNA-binding (Fig. 3*B, lanes 3* and *4*).

To further illustrate the specificity of the LT inhibitory effects on IRF3-mediated transcription, we used an IRF3-inducible luciferase construct, which displayed a robust induction in response to LPS stimulation (Fig. 4*A*). In accordance with the analysis of endogenous IRF3-controlled genes, the inducibility of the ISRE reporter by LPS was significantly inhibited after exposure of the cells to LT (Fig. 4*A*). IRF3 activation requires phosphorylation of five serine/threonine residues in its C terminus (9, 13). Substitution of these amino acids with the phosphomimetic aspartic acid (IRF3-5D) generates a constitutive active form of IRF3 capable of activating ISRE-controlled genes and reporter constructs (21). As anticipated, the expression of IRF3-5D yielded a vigorous activation of the ISRE luciferase. However, in contrast to the LPS-mediated induction, the response elicited by IRF3-5D was resistant to LT (Fig. 4*B*). Similarly, the luciferase production from an NFκB reporter plasmid was also unaffected by LT (data not shown).

To verify that cleavage of MKK3 or MKK6 accounts for the inhibitory effects of LT on ISG induction and to determine which MKK family member acts upstream of IRF3, we generated LF-resistant mutants of MKK3 and MKK6 by replacing

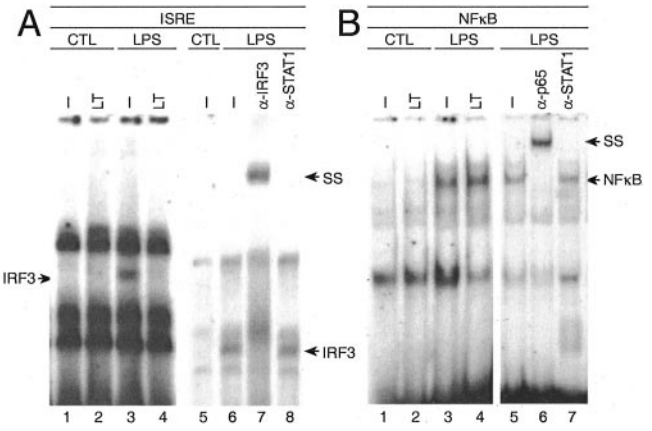

FIGURE 3. LT selectively inhibits IRF3 DNA-binding: *A,* Extracts described in Fig. 2 were analyzed by EMSA for IRF3 binding to the ISG54-ISRE. The identity of IRF3 was verified by supershift (SS) experiments using IRF3 or control antiserum. *B,* Same as *A,* except an NFκB-binding site was used as probe. Identity of the LPS-induced band as p65 was confirmed by supershifting with p65 Abs.

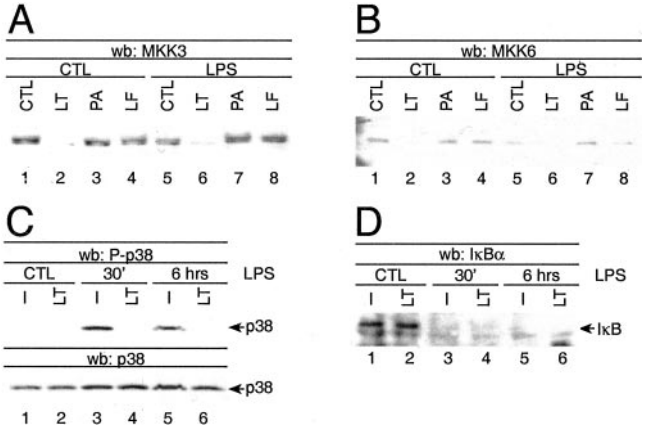

FIGURE 2. LT causes MKK3 and MKK6 cleavage and inhibits LPS-mediated phosphorylation of p38. Extracts were prepared from untreated and LPS-stimulated U373 cells that had been pre-exposed to PA, LF, or PA plus LF (= LT) for 8 h. Cell lysates were subject to Western blotting (wb) with Abs against MKK3 (*A*) or MKK6 (*B*). (*C*) Cell lysates were immunoblotted with Abs specific for P-p38 (*upper panel*) or total p38 (*lower panel*). *D,* The same lysates as in *A–C* were immunoblotted with Abs against IκB.

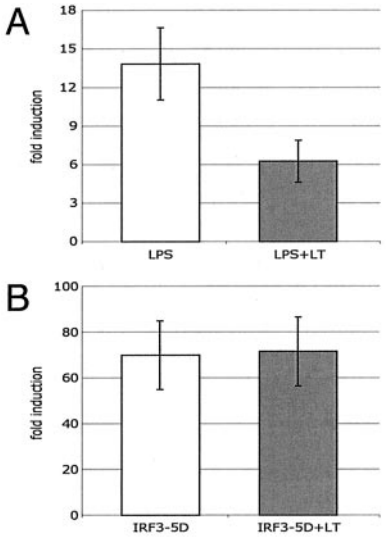

FIGURE 4. LT inhibits IRF3-mediated transcription. *A,* U373 cells were transiently transfected with ISRE-firefly luciferase and exposed to LT for 8 h before stimulation with LPS for 12 h. Firefly luciferase activity was normalized to cotransfected *Renilla* luciferase under the control of a thymidine kinase promoter. The results shown as LPS-mediated induction are the average of at least four independent transfections. *B,* Same as *A,* but cells were cotransfected with a plasmid encoding the constitutively active IRF3-5D. Results represent relative luciferase activity of at least four independent transfections.

Table I. *LF cleavage sites in MKKs*

Substrate Site	8	7	6	5	4	3	2	1	1′	2′	3′	4′	5′	6′	7′	8′
MEK1 (P8–I9)	M	P	K	K	K	P	T	P	**I**	Q	L	N	P	A	P	D
MEK2 (P10–A11)	A	R	R	K	P	V	L	P	**A**	L	T	I	N	P	T	I
MKK3b (R26–I27)	S	K	R	K	K	D	L	R	**I**	S	C	M	S	K	P	P
MKK6b (K14–I15)	K	K	R	N	P	G	L	K	**I**	P	K	E	A	F	E	Q

the isoleucine residues C-terminal to the cleavage site with alanine or aspartic acid, respectively (Table I. This approach had been used previously to generate MKK1 mutant proteins that resisted cleavage by LF in vitro (16). To test the susceptibility of these proteins toward LF, wild-type (wt) and mutant MKKs were in vitro translated, subject to incubation with increasing amounts of LF in vitro, and cleavage analyzed by immunoblotting using MKK3 and MKK6 antisera, respectively. As shown in Fig. 5*A,* wt MKK3 and MKK6 were completely cleaved during incubation with 100 μg of LF, whereas mutant MKK3(I27A) (Fig. 5*A, left, lanes 5* and *6*) and MKK6(I15D) (Fig. 5*A, right, lanes 5* and *6*) displayed only limited (MKK3) or no (MKK6) degradation.

After we had established the resistance of MKK3(I27A) or MKK6(I15D) toward LF-mediated cleavage, we generated stable U373 lines expressing these MKK mutants. Treatment of the selected cells with LT and subsequent immunoblotting of the resulting lysates with MKK3 antisera revealed the presence of MKK3 protein only in the cells expressing MKK3(I27A) (Fig. 5*B, left, lane 4*), but not in cells transfected with empty plasmid (Fig. 5*B, left, lane 2*) or MKK6(I15D) (Fig. 5*B, left, lane 6*). Conversely, MKK6 was detectable only after LT treatment of cells expressing MKK6(I15D) (Fig. 5*B, right, lane 6*), but not in the cells transfected with empty plasmid (Fig. 5*B, right, lane 2*) or MKK3(I27A) (Fig. 5*B, right, lane 4*). The apparent decrease in MKK3 or MKK6 levels in cells expressing MKK3(I27A) or MKK6(I15D), respectively, is the result of proteolytic cleavage of the endogenous, wt proteins (Fig. 5*B, left, lanes 3* and *4*; *right, lanes 5* and *6*), because the antisera detect both wt and mutant MKKs. Immunoblotting with epitope-directed Abs that specifically recognize the transfected protein confirmed the cleavage resistance of the mutant protein (data not shown).

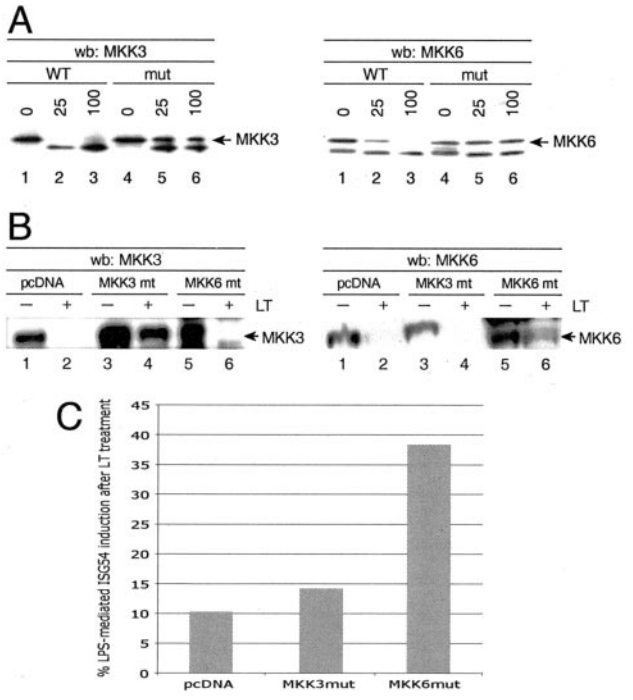

FIGURE 5. MKK6 mutant (mut, mt) resistant to LT cleavage protects IRF3-mediated transcription. *A,* Reticulocyte-translated wt or cleavage-resistant MKK3 (*left*) or MKK6 (*right*) were incubated with the indicated amounts of LF. Whole cell lysates were immunoblotted with MKK3 and MKK6 Abs, respectively. wb, Western blot. *B,* U373 cells stably expressing MKK3(I27A) (*lanes 2* and *3*) or MKK6(I15D) (*lanes 5* and *6*) were exposed to LT for 8 h, and whole cell lysates were immunoblotted for levels of LT-resistant MKK3 (*left*) or MKK6 (*right*) protein. *C,* U373 cell lines described in *B* were pretreated with LT for 8 h before stimulation with LPS for 6 h. ISG54 mRNA levels were analyzed by RNase protection assay, quantitated, and normalized to GAPDH mRNA levels. Bars indicate the level of LPS-mediated ISG54 induction after LT exposure relative to LPS-mediated ISG54 induction in the absence of the toxin (a representative result of three independent experiments is shown).

To determine which MKK mediates the ISG induction in response to LPS treatment, the cell lines described above were stimulated with LPS without or with prior exposure to LT, and the reduction in ISG54 expression was quantitated. In cells transfected with empty plasmid, LT treatment resulted in an ~90% inhibition in *ISG54* induction. The expression of MKK3(I27A) did not alter the extent to which LT blocked the LPS-mediated response. In contrast, cells expressing MKK6(I15D) were able to sustain a substantial up-regulation of *ISG54* in response to LPS even after LT exposure. This is particularly striking, as it appears that the relative amount of MKK6 protein present after LT treatment of cells expressing MKK6(I15D) is substantially less than the relative amount of cleavage-resistant MKK3 in MKK3(I27A)-transfected cells (Fig. 5*B*; *left,* compare *lanes 3* and *4*; *right*, compare *lanes 5* and *6*).

In summary, our findings demonstrate that inhibition of IRF3 activation accounts, at least in part, for the reduction in LPS-mediated cytokine production as a result of exposure to LT. Previous studies have established MKKs as targets for degradation by LF; however, the biological consequences of this event remained elusive as the cleavage of MKKs does not seem to correlate with LT-induced cell death.

IRF3 activation is a crucial part of the innate immune response as evidenced by the fact that IRF3-deficient mice fail to produce type I IFNs, as well as other proinflammatory cytokines and chemokines in response to TLR ligation (22, 23). Recent reports demonstrated a role for TBK1 and IKKϵ in the activation of IRF3 in response to viral infection or dsRNA (24, 25), which requires the phosphorylation of IRF3 on S396 (26). Importantly, phosphorylation of this residue does not occur in response to LPS (26), indicating that a distinct pathway mediates IRF3 activation via TLR2 and TLR4 ligands. Our results illustrate that MKK6-mediated activation of p38 is an essential component of the transcriptional activation of ISGs via IRF3 in response to TLR2/4 engagement. Furthermore, our findings also establish IRF3 as the first transcription factor to be subject to *B. anthracis* LT-mediated inhibition and provide a link between its biochemical actions and the attenuation of the innate immune response through prevention of the production of proinflammatory mediators.

Acknowledgments

We thank John Hiscott for the IRF3-5D construct, Jiahuai Han for the MKK3 and MKK6 reagents, Tadatsugu Taniguchi for the IRF3$^{-/-}$ mice, and Peter

Tobias and Gerald Feldman for kindly providing U373/CD14 cells and primary human monocytes, respectively. *B. anthracis* toxins were kindly provided by Karen Crawford of List Biological Laboratories.

References

1. Kopp, E. B., and R. Medzhitov. 1999. The Toll-receptor family and control of innate immunity. *Curr. Opin. Immunol. 11:13*.
2. Baldwin, A. S., Jr. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol. 14:649*.
3. Zhang, G., and S. Ghosh. 2000. Molecular mechanisms of NF-κB activation induced by bacterial lipopolysaccharide through Toll-like receptors. *J. Endotoxin Res. 6:453*.
4. Lee, J., L. Mira-Arbibe, and R. J. Ulevitch. 2000. TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide. *J. Leukocyte Biol. 68:909*.
5. Sanghera, J. S., S. L. Weinstein, M. Aluwalia, J. Girn, and S. L. Pelech. 1996. Activation of multiple proline-directed kinases by bacterial lipopolysaccharide in murine macrophages. *J. Immunol. 156:4457*.
6. Swantek, J. L., M. H. Cobb, and T. D. Geppert. 1997. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor α (TNF-α) translation: glucocorticoids inhibit TNF-α translation by blocking JNK/SAPK. *Mol. Cell. Biol. 17:6274*.
7. Goh, K. C., M. J. deVeer, and B. R. Williams. 2000. The protein kinase PKR is required for p38 MAPK activation and the innate immune response to bacterial endotoxin. *EMBO J. 19:4292*.
8. Navarro, L., K. Mowen, S. Rodems, B. Weaver, N. Reich, D. Spector, and M. David. 1998. Cytomegalovirus activates interferon immediate-early response gene expression and an interferon regulatory factor 3-containing interferon-stimulated response element-binding complex. *Mol. Cell. Biol. 18:3796*.
9. Lin, R., C. Heylbroeck, P. Pitha, and J. Hiscott. 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol. Cell Biol. 18:2986*.
10. Yoneyama, M., W. Suhara, Y. Fukuhara, M. Fukuda, E. Nishida, and T. Fujita. 1998. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J. 17:1087*.
11. Wathelet, M., C. H. Lin, B. S. Parekh, L. V. Ronco, P. M. Howley, and T. Maniatis. 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-β enhancer in vivo. *Mol. Cell 1:507*.
12. Navarro, L., and M. David. 1999. p38-dependent activation of interferon regulatory factor 3 by lipopolysaccharide. *J. Biol. Chem. 274:35535*.
13. Lin, R., Y. Mamane, and J. Hiscott. 1999. Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. *Mol. Cell. Biol. 19:2465*.
14. Lin, R., C. Heylbroeck, P. Genin, P. Pitha, and J. Hiscott. 1999. Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription. *Mol. Cell. Biol. 19:959*.
15. Duesbery, N. S., and G. F. Vande Woude. 1999. Anthrax toxins. *Cell Mol. Life Sci. 55:1599*.
16. Duesbery, N. S., C. P. Webb, S. H. Leppla, V. M. Gordon, K. R. Klimpel, T. D. Copeland, N. G. Ahn, M. K. Oskarsson, K. Fukasawa, K. D. Paull, and G. F. Vande Woude. 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science 280:734*.
17. Vitale, G., L. Bernardi, G. Napolitani, M. Mock, and C. Montecucco. 2000. Susceptibility of mitogen-activated protein kinase kinase family members to proteolysis by anthrax lethal factor. *Biochem. J. 352(Pt. 3):739*.
18. Pellizzari, R., C. Guidi-Rontani, G. Vitale, M. Mock, and C. Montecucco. 1999. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNγ-induced release of NO and TNFα. *FEBS Lett. 462:199*.
19. Orr, S. L., and P. Tobias. 2000. LPS and LAM activation of the U373 astrocytoma cell line: differential requirement for CD14. *J. Endotoxin Res. 6:215*.
20. Han, J., J. D. Lee, Y. Jiang, Z. Li, L. Feng, and R. J. Ulevitch. 1996. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J. Biol. Chem. 271:2886*.
21. Grandvaux, N., M. J. Servant, B. tenOever, G. C. Sen, S. Balachandran, G. N. Barber, R. Lin, and J. Hiscott. 2002. Transcriptional profiling of interferon regulatory factor 3 target genes: direct involvement in the regulation of interferon-stimulated genes. *J. Virol. 76:5532*.
22. Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity 13:539*.
23. Sakaguchi, S., H. Negishi, M. Asagiri, C. Nakajima, T. Mizutani, A. Takaoka, K. Honda, and T. Taniguchi. 2003. Essential role of IRF-3 in lipopolysaccharide-induced interferon-β gene expression and endotoxin shock. *Biochem. Biophys. Res. Commun. 306:860*.
24. Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis. 2003. IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol. 4:491*.
25. Sharma, S., B. R. tenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott. 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science 300:1148*.
26. Servant, M. J., N. Grandvaux, B. R. tenOever, D. Duguay, R. Lin, and J. Hiscott. 2003. Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. *J. Biol. Chem. 278:9441*.