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

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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. In innate immune recognition of bacterial infection is mediated by a system of germline-encoded receptors (Toll-like receptors; TLRs) 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 Zn²⁺-dependent metalloprotease, which cleaves short N-terminal fragments of several MKK isofoms (16, 17). This cleavage of MKKs does not appear to correlate with the 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 mAbs 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

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

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

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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.

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0022-1767/04/$02.00
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 NaVO₃, 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 32P-labeled probes corresponding to the ISRE of the ISG54 gene or the NFκB binding site. Lysates were incubated with poly(dexyinoisinate-deoxyctydylate) and labeled probes in binding buffer (40 mM KCl, 20 mM HEPES (pH 7.0), 1 mM MgCl₂, 0.1 mM EDTA, 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 antisera 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). 32P-labeled antisense riboprobes for IFN-activated gene (ISG) 54 and GAPDH were generated from the linearized plasmid using T7 or SP6 RNA polymerase. Labeled riboprobes 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 TaqPCR 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. 1A, 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-deficient mice and analyzed their transcriptional responses toward LPS. As shown in Fig. 1C, 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.

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 cells were exposed to LPS for 8 h before stimulation with LPS. Indeed, LPS treatment triggered a significant increase in RANTES mRNA levels (Fig. 1D, lane 2) which was almost completely inhibited by LT (Fig. 1D, 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. 1E, lane 2). However, pretreatment with LT abrogated this LPS-induced ISG54 expression (Fig. 1E, lane 3). Importantly, the IFN-β-mediated induction of ISG54 was unaffected by LT (Fig. 1E, 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. 2A) and MKK6 (Fig. 2B), 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. 2C, 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. 2C, 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. 2D, 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. 3A, lanes 3 and 6), as further verified by supershifts with IRF3 and control antisera (Fig. 3A, lanes 7 and 8). However, no IRF3/ISRE interaction was observed in cells exposed to LT before stimulation with LPS (Fig. 3A, 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. 3B, 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. 4A). 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. 4B). 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 phosmimetic 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.

**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 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 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.
We thank John Hiscott for the IRF3-5D construct, Jiahua Han for the MKK3 and MKK6 reagents, Tadatsugu Taniguchi for the IRF3 antibodies, and Peter T. Leary for help with Western blotting.
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.

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