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Signaling Pathways and Genes that Inhibit Pathogen-Induced Macrophage Apoptosis—CREB and NF-κB as Key Regulators

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Summary

Certain microbes evade host innate immunity by killing activated macrophages with the help of virulence factors that target prosurvival pathways. For instance, infection of macrophages with the TLR4-activating bacterium *Bacillus anthracis* triggers an apoptotic response due to inhibition of p38 MAP kinase activation by the bacterial-produced lethal toxin. Other pathogens induce macrophage apoptosis by preventing activation of NF-κB, which depends on IκB kinase β (IKKβ). To better understand how p38 and NF-κB maintain macrophage survival, we searched for target genes whose products prevent TLR4-induced apoptosis and a p38-dependent transcription factor required for their induction. Here we describe key roles for transcription factor CREB, a target for p38 signaling, and the plasminogen activator 2 (PAI-2) gene, a target for CREB, in maintenance of macrophage survival.

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Introduction

Macrophages are professional phagocytes that are key players in host defense against microbial infection (Aderem and Underhill, 1999). In addition to central depots in secondary lymphoid organs, macrophages reside in almost all tissues, serving as sentinels that detect microbial invaders. Upon recognition of pathogen-associated molecular patterns (PAMPs; Janeway and Medzhitov, 2002) or molecules released by damaged host cells, referred to as "danger signals" (Matzinger, 2002), macrophages produce cytokines, chemokines, and other inflammatory mediators that alert the remainder of the immune system to occurrence of infection and injury. Several distinct classes of receptor proteins participate in PAMP sensing and triggering of signaling cascades that culminate in expression of immune effector genes. Among such receptors, Toll-like receptors (TLR) were extensively studied in both vertebrates and invertebrates (Akira and Takeda, 2004; Beutler, 2004). Although different TLRs are activated by distinct agonists, their cytoplasmic domains share the ability to transduce PAMP-generated signals to the IκB kinase (IKK) and mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38. These effectors elicit target gene induction by modulating subcellular distribution, promoter recruitment, and transcriptional activity of specific transcription factors.

In addition to innate immune functions, TLR4 signaling can also trigger macrophage apoptosis under specific circumstances (Park et al., 2002, 2004; Hsu et al., 2004). The TLR4-mediated proapoptotic response requires activation of the dsRNA-responsive protein kinase PKR and inhibition of either p38 MAPK or IKK signaling. These findings suggest that progression of innate immune responses may be regulated at the level of macrophage survival. Of note, certain bacterial pathogens target TLR4-initiated antiapoptotic mechanisms to induce the death of activated macrophages and thereby evade detection and destruction by the host immune system. Bacterial toxins or virulence factors that participate in induction of macrophage apoptosis were shown to interfere with the IKK and/or MAPK effector modules of TLR4. For example, *Yersinia* YopP/J and *Salmonella* AvrA inhibit NF-κB activation (Collier-Hyams et al., 2002; Orth et al., 1999; Schesser et al., 1998). By contrast, the anthrax bacterium, *Bacillus anthracis*, induces apoptosis of TLR4-activated macrophages through its lethal toxin (LT), whose lethal factor (LF) catalytic subunit prevents p38 activation by cleavage of the upstream kinase MKK6 (Park et al., 2002).

Compromised macrophage survival is seen upon inactivation of either the IKKβ subunit of the IKK complex or p38 MAPK (Hsu et al., 2004). Whereas the transcription factor mediating the survival activity of IKKβ is NF-κB, the target for the survival activity of p38 MAPK is unknown. Based on the similar sensitivity of macrophages lacking either p38 or NF-κB to TLR4-induced apoptosis, we postulated that p38 may activate a tran-

scription factor that synergizes with NF-κB to induce transcription of gene(s) whose product(s) inhibits apoptosis. We therefore embarked on a search for such a transcription factor and target genes and found that the antiapoptotic p38 MAPK signal is relayed to the cAMP-responsive transcription factor CREB, which in turn cooperates with NF-κB to induce transcription of the gene encoding the serpin plasminogen activator inhibitor 2 (PAI-2). In conjunction with the Bcl-2 family protein Bfl-1/A1, whose expression also requires both p38 and NF-κB activation, PAI-2 maintains survival of TLR4-activated macrophages, illustrating unique antiapoptotic regulatory mechanisms in the TLR4 pathway.

Results

Survival of TLR4-Activated Macrophages Requires IKKβ and p38

Incubation of macrophages with *B. anthracis* LT sensitizes them to TLR4-dependent apoptosis through inhibition of p38 activation (Park et al., 2002). Although *B. anthracis* is a gram-positive bacterium that does not produce lipopolysaccharide (LPS), it activates TLR4 via its hemolysin-anthrolysin O (ALO) (Park et al., 2004). Besides p38, macrophage survival after TLR4 engagement requires activation of IKKβ (Hsu et al., 2004). These findings suggest that expression of crucial macrophage survival genes, whose products prevent TLR4-induced apoptosis, requires activation of both signaling pathways. To eliminate the possibility that the manner in which IKKβ has been inactivated influences the results, we used two different populations of IKKβ-deficient macrophages: fetal liver-derived macrophages (FLDMs) from *Ikkβ*$^{-/-}$ mouse embryos and bone marrow-derived macrophages (BMDMs) from *LysM-Cre/Ikkβ*$^{F/F}$ adult mice, referred to as *Ikkβ*$^{\Delta mye}$. *Ikkβ*$^{-/-}$ FLDMs did not express any IKKβ, whereas IKKα and IKKγ levels remained unaltered (Figure 1A). On the other hand, the extent of *Ikkβ* deletion and IKKβ levels in bulk BMDM cultures derived from *Ikkβ*$^{\Delta mye}$ mice varied to a substantial degree (data not shown). Among independently generated BMDMs, we used a population that exhibited extensive (90%–95%) deletion of the floxed *Ikkβ*^{F} allele, such that most cells did not contain IKKβ (Figure 1A).

We first checked the sensitivity of these cells to apoptosis caused by infection with the Sterne strain of *B. anthracis*. We previously showed that the *B. anthracis*-induced macrophage apoptosis requires functional TLR4 (Hsu et al., 2004), which is activated by ALO (Park et al., 2004). Wild-type (wt) and *Ikkβ*$^{-/-}$ FLDMs underwent apoptosis following *B. anthracis* infection (Figure 1B). Preincubation with In-2-LF, a cell-permeable inhibitor of LF protease activity, prevented *B. anthracis*-induced apoptosis of wt but not *Ikkβ*$^{-/-}$ FLDMs (Figure 1B), indicating that macrophage survival requires two nonredundant signaling components: the LF-sensitive p38 MAPK and the LF-insensitive IKKβ.

We substantiated the antiapoptotic functions of IKKβ and p38 MAPK by determining their effect on the kinetics of TLR4-induced macrophage apoptosis. Incubation of wt or *Ikkα*$^{AA/AA}$ (derived from mice harboring a nonactivatable allele of *Ikkα* [Cao et al., 2001]) BMDMs with LPS did not result in much apoptotic cell death within the first 24 hr, but TLR4 engagement in *Ikkβ*$^{\Delta mye}$ BMDMs caused a robust and rapid apoptotic response detected as early as 6 hr after LPS addition (Figure 1C). The kinetics of TLR4-mediated apoptosis in *Ikkβ*$^{\Delta mye}$ BMDMs were very similar to those found in wt BMDMs whose p38 activity was inhibited by either LT or the small-molecule inhibitor SB202190 (Figure 1D). Incubation of macrophages lacking either IKKβ or p38 activity, but not wt BMDMs, with ALO also resulted in extensive apoptosis (see Figure S1A in the Supplemental Data available with this article online). Importantly, incubation of BMDMs with ALO has the same consequences as their incubation with LPS: resulting in cytokine induction (Park et al., 2004) or apoptosis when combined with SB202190 or LT (Figure S1B). To substantiate the survival function of p38, we generated BMDMs from *LysM-Cre/p38α*$^{F/F}$ (*p38α*$^{\Delta mye}$) mice and stimulated them with LPS. A *p38α*$^{\Delta mye}$ BMDM preparation with more than 90% deletion of the floxed *p38α*^{F} allele and a comparable reduction in p38 activity exhibited normal levels of ERK and JNK activation and IκBα degradation but was sensitive to LPS-induced apoptosis just like LT- and SB202190-treated macrophages (Figure S2). This experiment also underscores the role of the p38α isoform, one of the two SB202190-inhibitable p38 MAPK isozymes, in survival of TLR4-activated macrophages.

Next we examined LPS-induced NF-κB activation in *Ikkβ*$^{-/-}$ FLDMs and *Ikkβ*$^{\Delta mye}$ BMDMs. Both types of IKKβ-deficient macrophages exhibited a nearly complete defect in LPS-induced nuclear translocation of c-Rel, whereas nuclear translocation of RelA/p65 was reduced by approximately 50% (Figure 1E). Importantly, the p38 inhibitor SB202190 (Figure 1E) or LT (data not shown) did not affect the LPS-induced nuclear translocation of either RelA/p65 or c-Rel.

Identification of p38-Dependent NF-κB Target Genes in Macrophages

As p38 appears to have its own regulatory targets rather than directly affecting NF-κB activation, we sought to identify a transcription factor that mediates the survival function of p38 in TLR4-activated macrophages. To this end, we examined the promoter sequence of genes induced by LPS in an IKKβ- and p38-dependent manner and searched for common transcription factor binding sites. First, we performed a microarray experiment with RNAs isolated from resting and LPS-stimulated J774A.1 macrophage cells. Approximately 1.5% of the approximately 8000 microarray-analyzed genes were found to be LPS inducible (data not shown). We then used quantitative real-time PCR (Q-PCR) to confirm the LPS inducibility of these genes in BMDMs.

Of the LPS-inducible genes tested by Q-PCR, approximately 66% were dependent on IKKβ, as their induction was substantially reduced in IKKβ-deficient macrophages (Figure 2A). Next we examined which of the IKKβ-dependent genes were affected by inhibition of p38 MAPK in wt macrophages. Only 57% of the IKKβ-dependent genes exhibited some p38 dependence (Figure 2A; Figure S3). This analysis resulted in identification of 14 NF-κB target genes (based on IKKβ dependence) whose induction by LPS also required

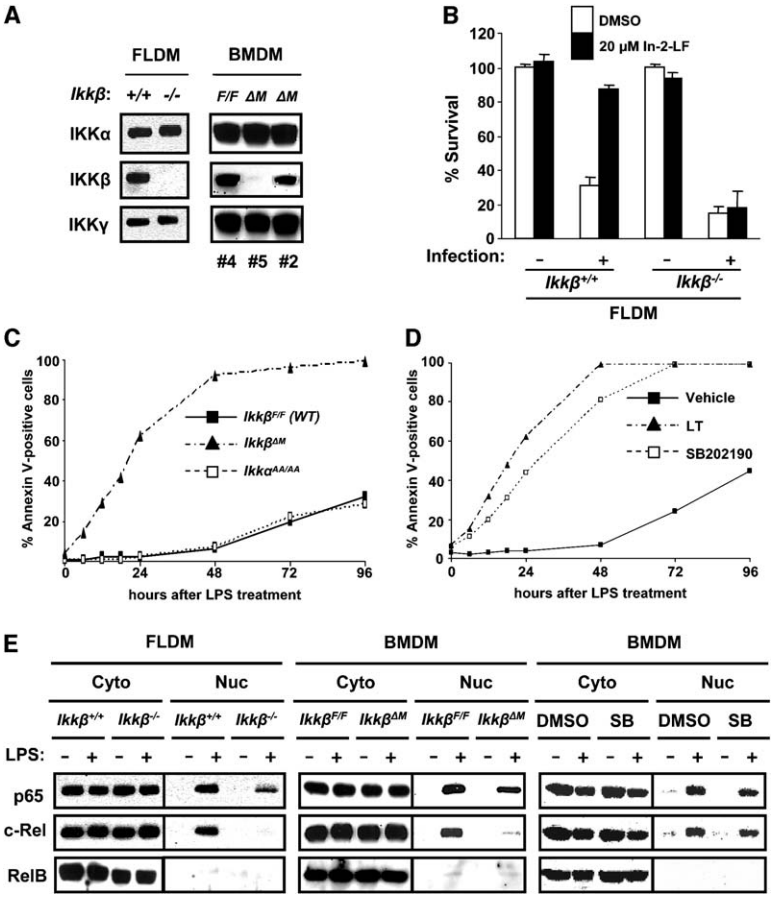


Figure 1. The IKKβ/NF-κB and p38 MAPK Signaling Modules Are Required for Survival of TLR4-Activated Macrophages

(A) Whole-cell lysates from *Ikkβ*$^{+/+}$ and *Ikkβ*$^{-/-}$ FLDMs or *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta mye}$ (ΔM) BMDMs were analyzed by immunoblotting with antibodies to IKK subunits. *Ikkβ*$^{\Delta mye}$ BMDMs from line #5 are IKKβ deficient.

(B) *Ikkβ*$^{+/+}$ and *Ikkβ*$^{-/-}$ FLDMs were infected with *B. anthracis* at moi = 0.1 in the absence or presence of 20 μM In-2-LF. After 6 hr, cell viability was measured by H33258 staining. Results are expressed as percent cell survival, with noninfected *Ikkβ*$^{+/+}$ cells showing 100% survival. The data represent the average ± SD for three independent experiments.

(C) *Ikkβ*$^{F/F}$, *Ikkβ*$^{\Delta mye}$ (*Ikkβ*$^{\Delta M}$; line #5), and *Ikkα*$^{AA/AA}$ BMDMs were treated with LPS (100 ng/ml). At different time points, the proportion of apoptotic cells was determined by Annexin V staining.

(D) BMDMs (C57BL/6J) were preincubated with DMSO (0.1%; Vehicle), LT (500 ng/ml of LF + 1 μg/ml of PA), or SB202190 (10 μM) for 2 hr, and then treated with LPS (100 ng/ml) and analyzed as in (C).

(E) Macrophages of the indicated *Ikkβ* genotypes were left untreated or treated with LPS for 30 min. Immunoblot analysis was performed on cytoplasmic (Cyto) and nuclear (Nuc) extracts with antibodies against NF-κB subunits. Where indicated, DMSO and SB202190 (SB) were added 2 hr before LPS.

p38 activation (Figure 2B and data not shown). These genes included one well-established antiapoptotic gene (*Bfl-1/A1*) and several genes (such as *Il-1β*, *Cox-2*, *Il-6*, and *Vcam-1*) without an obvious antiapoptotic function.

Comparison of the promoter organization of p38-dependent and -independent genes revealed that consensus binding sites for basic-region leucine zipper (bZIP) transcription factors, such as AP-1, C/EBP, and ATF/CREB, were enriched in p38-dependent gene regulatory regions (2.4 versus 0.67 sites per promoter; Figure 2C, Figure S4, and data not shown). Certain bZIP transcription factors, including ATF-2 (Raingeaud et al., 1995), CHOP/GADD153 (Wang and Ron, 1996), and CREB (Tan et al., 1996; Yu et al., 2001), are activated by p38 MAPK. Such factors may therefore mediate the p38 responsiveness of these genes.

CREB as a p38 MAPK-Regulated Transcription Factor Required for Macrophage Survival

To identify the transcription factor targeted by p38 in macrophages, we examined phosphorylation of individual bZIP transcription factors as well as nuclear translocation of NF-κB proteins in response to TLR engagement. bZIP transcription factors activated by phosphorylation were first analyzed in the nuclear fractions of RAW264.7 macrophages with antibodies specific to their phosphorylated and active form (Figures 3A and 3B). This analysis revealed that CREB, c-Jun, and ATF-2 were transiently activated in response to LPS. To test p38 MAPK dependence, RAW264.7 cells were preincubated with SB202190 before LPS stimulation. Among the bZIP proteins thus examined, only CREB activation was blocked by the p38 inhibitor as was previously found (Figure 3A). The p38 dependence of LPS-induced CREB activation has been also shown by others in another macrophage cell line, J774A.1 (Bradley et al., 2003). We confirmed that BMDMs incubated with either SB202190 or anthrax LT did not activate CREB in response to LPS (Figure 3C).

CREB is activated through phosphorylation at serine 133 (S133) in response to stimuli, which act through either protein kinase A (PKA) or other protein kinases (Mayr and Montminy, 2001). The amino acid sequence surrounding CREB S133 does not fit the consensus recognized by MAPKs. Most likely, p38 activates another protein kinase that phosphorylates CREB. Among known CREB S133 kinases, MSK1 and MSK2 are activated by p38 in fibroblasts (Deak et al., 1998). To examine their role in p38-dependent CREB phosphorylation in macrophages, we derived BMDMs from mice lacking both MSK1 and MSK2 (Wiggin et al., 2002). LPS-induced CREB phosphorylation in these double-knockout BMDMs was severely diminished (Figure 3D), suggesting that MSK1 and MSK2 are the major subordinate kinases linking p38 to CREB activation in TLR4-activated macrophages.

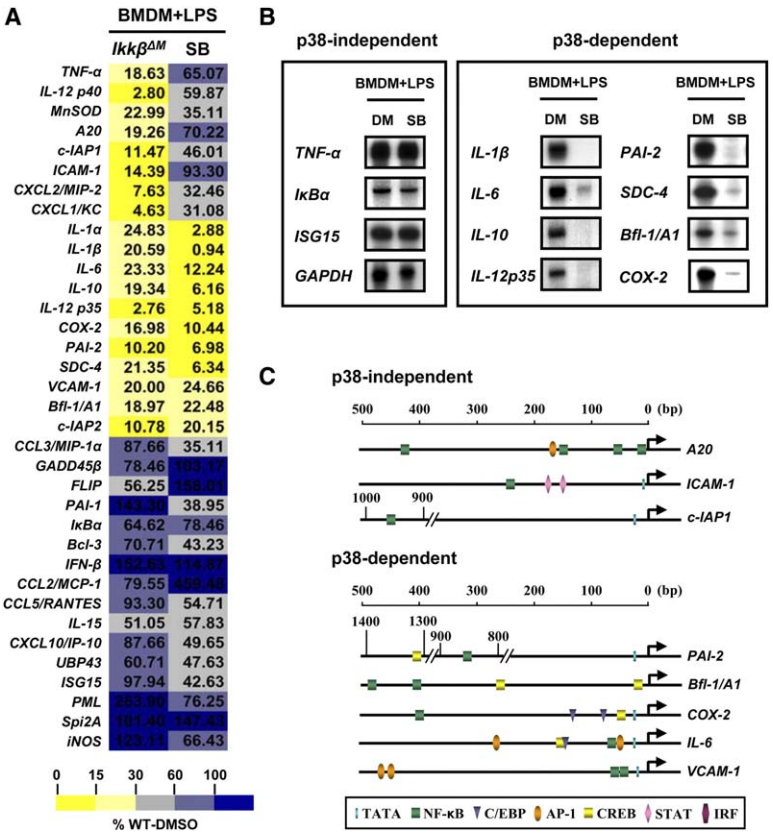


Figure 2. Identification of LPS-Inducible NF-κB Target Genes that Require p38 Activity

(A) Expression of 35 LPS-inducible genes in *Ikkβ*$^{\Delta mye}$ BMDMs (*Ikkβ*$^{\Delta M}$) and SB202190-treated C57BL/6J BMDMs (SB), at 4 hr poststimulation, was analyzed by real-time PCR. The values represent percentage of mRNA in these samples relative to control samples (*Ikkβ*$^{F/F}$ BMDMs and DMSO-treated C57BL/6J BMDMs, respectively).

(B) BMDMs were stimulated with LPS in the absence (DM-DMSO) or presence of SB202190 (SB). After 4 hr, expression of the indicated genes was analyzed by RNase protection.

(C) Promoter organization of p38-independent and -dependent NF-κB target genes. DNA sequences were obtained from the GenBank and Celera genomic databases and analyzed using TESS software (http://www.cbil.upenn.edu/tess/). Transcription factor binding site sequences for the eight genes are listed in Figure S4.

We next examined whether CREB mediates survival of TLR4-activated macrophages. As gene disruption failed to completely abolish CREB activity due to functional redundancy with the closely related bZIP proteins ATF1 and CREM (Hummler et al., 1994), we used a highly specific dominant-negative CREB molecule, ACREB, that inhibits the function of all CREB-related proteins but has no effect on other bZIP transcription factors (Ahn et al., 1998). Infection of BMDMs with an ACREB lentivirus, but not with a control lentivirus expressing green fluorescent protein (GFP), resulted in a small increase in basal apoptosis that was clearly enhanced by LPS, which had only a marginal effect on viability of BMDMs infected with the GFP lentivirus (Figures 3E and 3F).

Anthrax Edema Factor Promotes CREB Activation and Macrophage Survival

Since inhibition of CREB function sensitizes macrophages to LPS-induced apoptosis, we examined whether CREB activation through an alternative pathway can prevent TLR4-triggered apoptosis of macrophages whose p38 pathway has been blocked. To activate CREB in a p38-independent manner, we employed two agents that elevate intracellular cAMP: anthrax ET, a bacterial toxin whose catalytic subunit, edema factor (EF), has adenylyl cyclase (AC) activity (Collier and Young, 2003); and forskolin, an activator of endogenous AC (Seamon et al., 1981). Elevation of cAMP is expected to activate PKA, the first identified CREB S133 kinase (Gonzalez and Montminy, 1989). Treatment of BMDMs with either ET or forskolin induced CREB S133 phosphorylation (Figure 4A). CREB activation by ET was not blocked by LT and took place also in MSK1/MSK2-deficient BMDMs (Figure 4B). Thus, unlike LPS, cAMP activates CREB independently of p38 MAPK. Furthermore, ET activated CREB in LPS-stimulated macrophages in which p38 signaling was blocked by LT (Figure 4C). Importantly, CREB activation by ET was accompanied by enhanced survival of LT-exposed macrophages (Figures 4D and 4E). Cell-permeable cAMP analogs also protected LT-treated macrophages from TLR4-induced apoptosis (Figure 4E). The survival-enhancing activity of ET required CREB, as ET did not enhance survival of ACREB-expressing BMDMs that were incubated with LPS (Figure 4F). Hence, CREB is a point of convergence for at least two antiapoptotic signaling pathways in macrophages, dependent upon either p38 MAPK or cAMP. These findings raise the interesting possibility that *B. anthracis* may control the timing of macrophage apoptosis by modulating expression of its two toxin catalytic subunits LF and EF.

PAI-2: A Transcriptional Target for p38, CREB, and NF-κB

Having identified CREB as an antiapoptotic and p38-responsive transcription factor in activated macrophages, we searched for a transcriptional target for CREB that could act as a survival factor. Based on the finding that most p38-dependent LPS-inducible genes

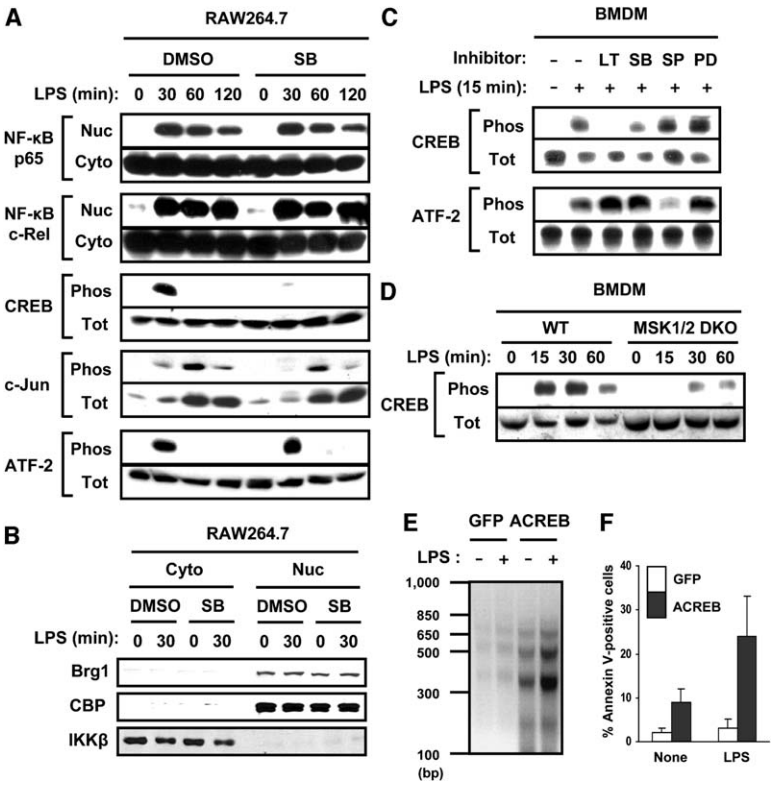


Figure 3. CREB Is a p38-Dependent Transcription Factor Needed for Inhibition of TLR4-Induced Macrophage Apoptosis

(A) RAW264.7 macrophages were treated with LPS for the indicated duration in the presence of either DMSO or SB202190, after which cytoplasmic (Cyto) and nuclear (Nuc) extracts were prepared and analyzed by immunoblotting with the antibodies indicated on the left. Antibodies specific to the phosphorylated form (Phos) of the proteins were used along with antibodies that recognize all protein forms (Tot).

(B) The extracts used in (A) were analyzed by immunoblotting with the antibodies that detect cytoplasmic (IKKβ) and nuclear (Brg1 and CBP) proteins.

(C) BMDMs were preincubated with LT (500 ng/ml LF + 1 μg/ml PA), SB202190 (SB; 10 μM), SP600125 (SP; 20 μM), and PD98059 (PD; 10 μM) for 2 hr, and then treated with LPS (100 ng/ml) for 30 min. Whole-cell lysates were prepared and analyzed by immunoblotting as in (A).

(D) BMDMs from wt and *Msk1/Msk2* double knockout mice (DKO) were analyzed as in (A).

(E and F) BMDMs were infected with lentiviruses expressing GFP or ACREB. After 48 hr, the cells were left unstimulated or stimulated with LPS (100 ng/ml) for 24 hr. The extent of apoptosis was determined by DNA fragmentation and gel electrophoresis (E) or annexin V staining (F). The data represent the average ± SD for three independent experiments.

in macrophages also require IKKβ (Figure 2A), we postulated that such a target should also be dependent on NF-κB. We therefore identified several p38-dependent NF-κB target genes whose promoters contained obvious CREB binding sites (Figure 2C) and examined them in further detail. The results described below pointed to *Pai-2*, which encodes an endogenous serine protease inhibitor, a member of the serpin family (Silverman et al., 2001), as a likely candidate. Among the three serpins that are most strongly induced upon LPS treatment of BMDMs (Figure 5A), PAI-2 is the only one whose induction requires both IKKβ and p38 (Figures 5B and 5C). Importantly, inhibition of CREB activity prevented induction of *Pai-2* mRNA by LPS but had no effect on induction of another p38- and NF-κB-dependent survival gene: *Bfl-1/A1* (Figure 5D). SB202190 and ACREB also inhibited induction of PAI-2 protein in BMDMs stimulated with LPS (Figure 5E) or ALO (Figure S1C). When LT-induced inhibition of CREB phosphorylation in LPS-stimulated macrophages was reversed by ET (Figure 4C), induction of *Pai-2* but not other p38-dependent antiapoptotic genes was significantly restored (Figure 5F).

To confirm that *Pai-2* is a transcriptional target for NF-κB and CREB, we carried out chromatin immunoprecipitation (ChIP) experiments. *Pai-2* contains single putative NF-κB and CREB binding sites at –860 bp and –1319 bp, respectively, within its control region (Figure 2B). The ChIP analysis revealed that CREB was efficiently recruited to the *Pai-2* regulatory region even prior to LPS stimulation (Figure 5G). By contrast, CREB recruitment could not be detected at the *iNos* and *IκBα* promoters, whose activation was insensitive to ACREB (data not shown). Although CREB was constitutively present at the *Pai-2* promoter, S133 phosphorylation of promoter bound CREB was upregulated by LPS and required p38 activity (Figure 5G). Basal phosphorylation of CREB on the *Pai-2* promoter prior to LPS stimulation was found to originate from M-CSF signaling as it disappeared upon withdrawal of M-CSF from the culture medium (Figure S5). The NF-κB proteins RelA/p65 and c-Rel were recruited to the *Pai-2*, *IκBα*, and *iNos* promoters only after LPS stimulation (Figure 5G). Occupancy of the *Pai-2* promoter by NF-κB and phosphorylated CREB was accompanied by increased Pol II recruitment, indicative of enhanced initiation of transcription (Figure 5G). Although inhibition of p38 blocked phosphorylation of promoter bound CREB without affecting CREB recruitment, it did inhibit recruitment of NF-κB proteins, and consequently Pol II, to the *Pai-2* promoter (Figure 5G). By contrast, inhibition of p38 had no effect on recruitment of NF-κB proteins or Pol II to the *iNos* and *IkBα* regulatory regions.

PAI-2 Inhibits Apoptosis of TLR4-Activated Macrophages

Having identified *Pai-2* as a common transcriptional target for several survival pathways, we examined its antiapoptotic function in TLR4-activated macrophages. Previously, PAI-2 was reported to antagonize mycobacterial and viral cytopathic effects in human macrophages and carcinoma cell lines (Gan et al., 1995; An-

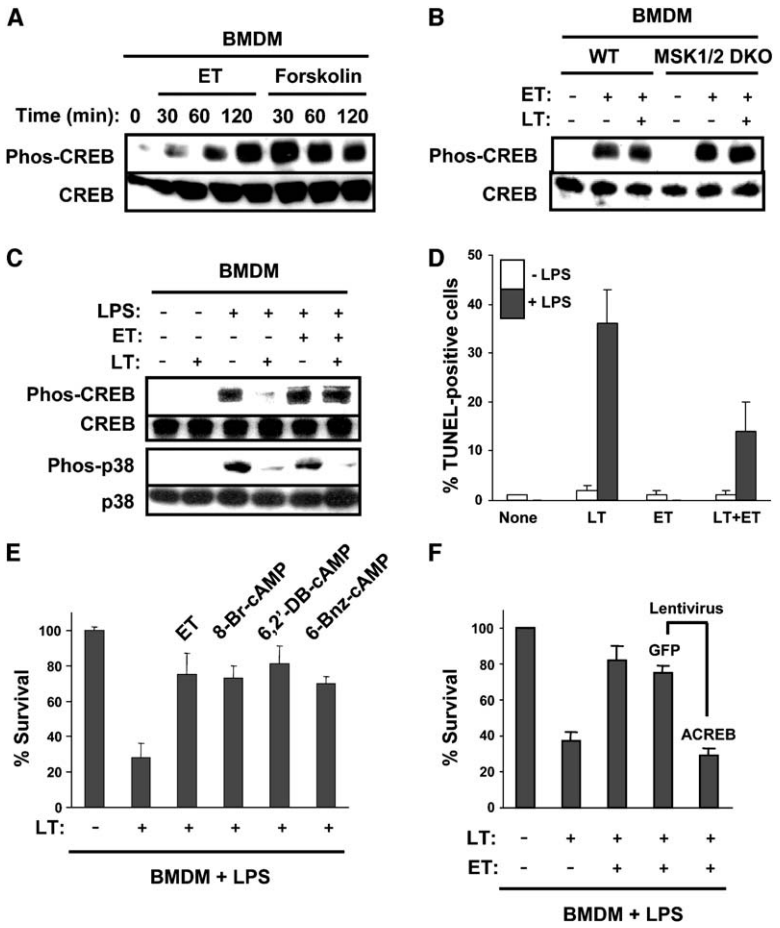


Figure 4. cAMP Signaling Promotes Macrophage Survival by Restoring CREB Activation Independently of p38

(A) BMDMs were treated with ET or forskolin (100 μM) for the indicated duration. Whole-cell lysates were prepared and analyzed by immunoblotting.

(B) BMDMs from wt and *Msk1*/*Msk2* double knockout mice (DKO) were preincubated without or with LT for 2 hr and then stimulated with ET (500 ng/ml of EF + 1 μg/ml PA) for 2 hr. Whole-cell lysates were prepared and analyzed as in (A).

(C) BMDMs were preincubated without or with ET and LT for 2 hr as indicated and then treated or not with LPS for 30 min. Whole-cell lysates were prepared and analyzed as in (A).

(D) BMDMs were preincubated without or with ET and LT as in (C) and then treated with LPS. After 24 hr, apoptotic macrophages were scored by a TUNEL assay.

(E) BMDMs were preincubated with ET, LT, and/or various cAMP analogs (100 μM) as indicated for 2 hr and then treated with LPS. After 24 hr, cell viability was measured.

(F) BMDMs were left uninfected or infected with lentiviruses expressing GFP or ACREB. After 48 hr, cells were preincubated without or with LT and ET as indicated and stimulated with LPS as in (D) and cell viability was measured. The data in (D)–(F) represent the average ± SD for three independent experiments.

talis et al., 1998). Other studies showed that PAI-2 inhibits TNFα-induced apoptosis of human carcinoma and fibrosarcoma cell lines (Dickinson et al., 1995; Kumar and Baglioni, 1991), but we could not detect enhanced TNFα-induced apoptosis in p38α-deficient fibroblasts, although these cells did not respond to TNFα with *Pai-2* induction (data not shown). The first hint for an antiapoptotic function for PAI-2 in TLR4-activated macrophages was obtained by screening different chemical inhibitors for their ability to enhance survival of LPS-stimulated and p38-inhibited macrophages. In these experiments, the serine protease inhibitor N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) protected p38-inhibited macrophages from LPS-induced apoptosis (Figure S6). More direct evidence for the survival function of PAI-2 was provided by analyzing macrophages derived from the human promonocytic leukemia cell THP-1, which carries a naturally occurring mutation in the *PAI-2* gene (Katsikis et al., 2000). Although THP-1-derived macrophages (TDMs) respond to TLR4 engagement with p38 and IKK activation and cytokine gene induction (data not shown), they are highly susceptible to LPS-induced apoptosis (Figure 6A). Unlike the situation in BMDMs and RAW264.7 macrophages, TLR4-induced apoptosis in TDMs does not require p38 inhibition (Figure S7). To address whether the increased susceptibility to TLR4-induced apoptosis is due to PAI-2 deficiency, we generated THP-1 derivatives stably expressing PAI-2. Such cells were resistant to LPS-induced apoptosis (Figure 6A). We also transduced TDMs with adenoviral vectors and found that TLR4-induced apoptosis was inhibited by PAI-2 but not PAI-1 (Figure 6B). These data support the hypothesis that *PAI-2* is an important survival gene in TLR4-activated human macrophages.

We extended our analyses to mouse BMDMs with a targeted deletion of the *Pai-2* gene (Dougherty et al., 1999). Consistent with the results described above, *Pai-2*$^{-/-}$ BMDMs exhibited a stronger apoptotic response to LPS alone in comparison to wt BMDMs (Figure 6C). However, this response was not as robust as the one seen in IKKβ-deficient macrophages or wt macrophages incubated with SB202190, suggesting that other antiapoptotic genes that depend on both pathways, for instance *Bfl-1/A1*, provide protection in the absence of PAI-2. To address this possibility, we transfected RAW264.7 macrophages with various antiapoptotic genes and tested their ability to prevent apoptosis induced by treatment with LPS and SB202190. Expression of either Bfl-1/A1 or PAI-2 enabled the transfected cells to survive selection in a medium containing both LPS and SB202190 (Figures 6D and 6E). Treatment with either LPS or SB202190 alone under the same condition did not significantly reduce the viability of RAW264.7 macrophages (data not shown). This pro-

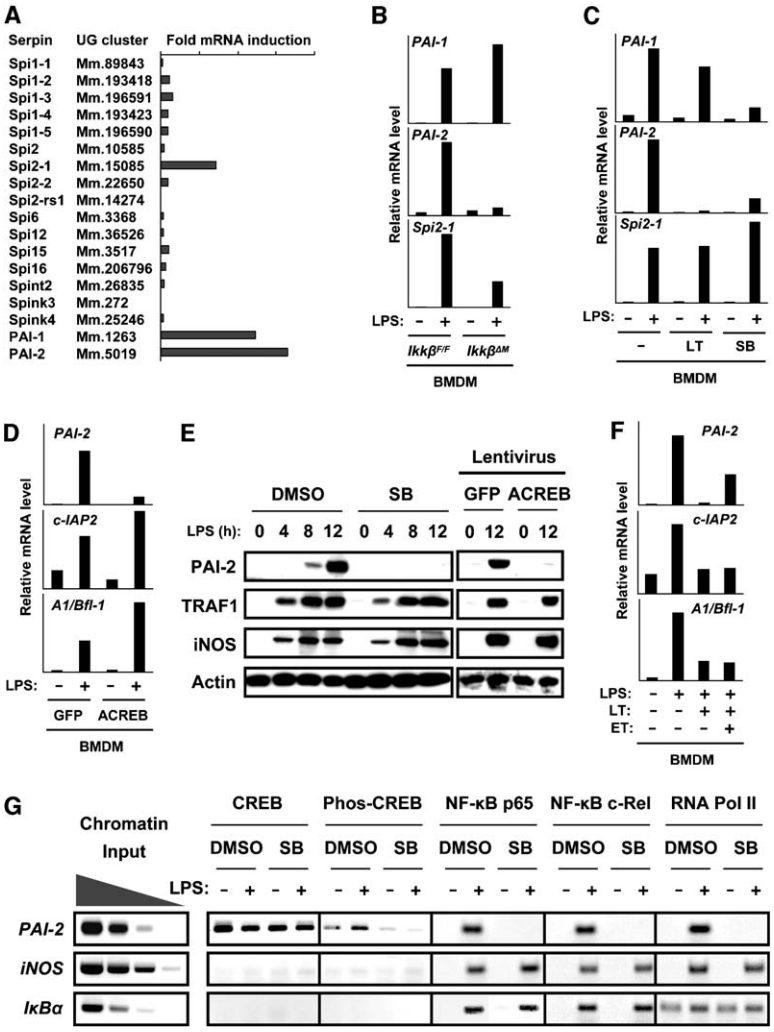


Figure 5. TLR4-Mediated PAI-2 Induction Depends on the IKKβ/NF-κB and p38/CREB Modules

(A) Induction of different serpin mRNAs in LPS-stimulated BMDMs was determined by a DNA microarray experiment.

(B–D) Total RNA was isolated from the following cells and relative expression of various mRNAs was determined by real-time PCR. (B) *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta mye}$ (*Ikkβ*$^{\Delta M}$) BMDMs left unstimulated or stimulated with LPS for 4 hr. (C) Wild-type BMDMs preincubated with DMSO LT or SB202190 for 2 hr, and then treated with LPS 4 hr. (D) Wild-type BMDMs infected with lentiviruses expressing GFP or ACREB, and then left unstimulated or stimulated with LPS for 4 hr.

(E) BMDMs were preincubated with DMSO or SB202190 for 2 hr (left) or infected with lentiviruses expressing GFP or ACREB for 48 hr (right). Cells were then treated with LPS for the indicated duration. Whole-cell lysates were prepared and immunoblotted with the indicated antibodies.

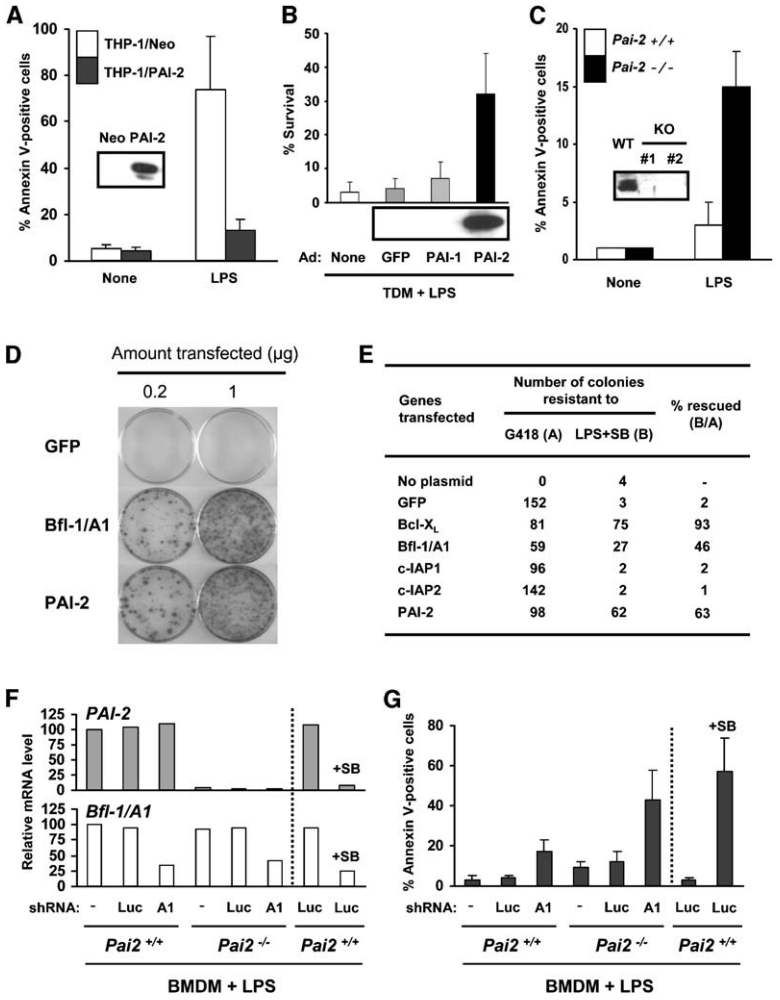
(F) BMDMs were preincubated or not with LT and/or ET for 2 hr, and then treated with LPS for 4 hr. Relative expression of various mRNAs was determined as in (B).

(G) BMDMs were preincubated with DMSO or SB202190 for 2 hr and then left untreated or treated with LPS before being fixed with formaldehyde. Chromatin was prepared, sonicated, and immunoprecipitated with the indicated antibodies for ChIP analysis. Recruitment of the different transcription factors and the large subunit of RNA polymerase II (Pol II) was determined by PCR analysis with primer sets specific to the indicated gene promoters.

tective effect was also seen with other Bcl-2 family members, such as Bcl-2 (data not shown) and Bcl-X_L, but not with inhibitor of apoptosis (IAP) family members (Figure 6E). Among Bcl-2 family members that conferred apoptotic resistance to LPS+ SB202190-treated macrophages, only Bfl-1/A1 expression depends on both the IKK/NF-κB and p38 pathways (Figures 2A and 2B; Figure S8). Therefore, the importance of Bfl-1/A1 was further examined through loss-of-function experiments. As functional Bfl-1/A1 protein is encoded by three clustered genes in the mouse genome (Hatakeyama et al., 1998), we performed RNA interference (RNAi) with a lentivirus expressing a short hairpin (sh) RNA that can knock down expression of all three Bfl-1/A1 isoforms. In both wt and *Pai-2*$^{-/-}$ BMDMs, infection with the A1 shRNA-containing lentivirus caused more than 3-fold decrease in *Bfl-1/A1* mRNA amount, similar to the effect of the p38 inhibitor SB202190 (Figure 6F). The knockdown of Bfl-1/A1 sensitized both wt and *Pai-2*$^{-/-}$ BMDMs to TLR4-induced apoptosis, with the effect on *Pai-2*$^{-/-}$ BMDMs similar to that of SB202190 on wt macrophages (Figure 6G). These results strongly suggest that *Pai-2* and *Bfl-1/A1* are critical p38-dependent antiapoptotic genes for preventing apoptosis of TLR4-activated macrophages.

Discussion

Our previous (Park et al., 2002; Hsu et al., 2004) and present studies define a critical innate immunity-associated antiapoptotic response responsible for survival of TLR4-activated macrophages (Figure 7). The new findings described above indicate that in the antiapoptotic branch of TLR4 signaling, the IKK/NF-κB and p38 MAPK modules cooperate to induce transcription of two critical antiapoptotic genes, *Pai-2* and *Bfl-1/A1*, whose products block the concurrent activation of the proapoptotic pathway. Unless opposed, this proapoptotic pathway can bring about rapid macrophage death and premature cessation of the innate immune response. The ability of PAI-2 to inhibit TLR4-induced macrophage apoptosis suggests a novel apoptotic mechanism that is regulated by a serpin family member. An antiapoptotic function for a serpin, however, is not unprecedented. It was shown that the serpin Spi2A/Spi2-1 promotes survival of cytotoxic T lymphocytes



Genes transfected	Number of colonies resistant to G418 (A)	Number of colonies resistant to LPS+SB (B)	% rescued (B/A)
No plasmid	0	4	-
GFP	152	3	2
Bcl-X_L	81	75	93
Bfl-1/A1	59	27	46
c-IAP1	96	2	2
c-IAP2	142	2	1
PAI-2	98	62	63

Figure 6. PAI-2 Protects Macrophages from LPS-Induced Apoptosis

(A) Macrophages derived from THP-1 cells stably transfected with either empty (Neo; open bars) or PAI-2 (solid bars) expression vectors were treated with LPS. After 24 hr, the percentages of apoptotic cells were determined by staining with annexin V. The inset shows the level of PAI-2 expression.

(B) THP-1 cells infected with adenoviruses expressing GFP, PAI-1, or PAI-2 were differentiated into macrophages and stimulated with LPS. After 24 hr, cell viability was measured. Inset: PAI-2 expression.

(C) *Pai-2*$^{+/+}$ (wt) and *Pai2*$^{-/-}$ (KO) BMDMs were left untreated or treated with LPS for 48 hr. The percentages of apoptotic cells were determined as in (A). Inset: PAI-2 expression in two different batches of KO BMDMs.

(D) RAW264.7 macrophages transfected with the indicated amounts of GFP, Bfl-1/A1, or PAI-2 expression vectors were cultured in the presence of LPS plus SB202190. After 14 days, viable cells were visualized by Giemsa-Wright staining.

(E) RAW264.7 macrophages were transfected with the indicated expression vectors. After 2 days, transfected cells were divided into two culture dishes and cultured further in the presence of G418 (1 mg/ml) or LPS (100 ng/ml) plus SB202190 (20 μM), respectively. After 14 days, viable colonies were visualized as in (D) and counted.

(F and G) *Pai-2*$^{+/+}$ (wt) and *Pai2*$^{-/-}$ (KO) BMDMs were infected with lentiviruses expressing: no shRNA (–), luciferase-specific shRNA (Luc), or *Bfl-1/A1* (A1)-specific shRNA, and then stimulated with LPS for 24 hr. Relative expression of *Pai-2* and *Bfl-1/A1* mRNAs (F) was determined by real-time PCR. The percentages of apoptotic cells (G) were determined as in (A). For comparison, wt BMDMs infected with the Luc lentivirus were preincubated with DMSO or SB202190 before LPS stimulation and analyzed in parallel. The data in (A)–(C) and (G) represent the average ± SD for three independent experiments.

during differentiation into memory CD8 T cells (Liu et al., 2004). Moreover, the cowpox virus *crmA* gene, which codes for a serpin (Ray et al., 1992), suppresses apoptosis in a variety of cell types (Gagliardini et al., 1994; Enari et al., 1995; Tewari et al., 1995). However, it is unclear whether these serpins share regulatory targets and act through similar mechanisms.

Although different in mechanism, macrophage apoptosis triggered by TLR4 is conceptually reminiscent of responses elicited by type 1 TNF receptor (TNFR1) and the T cell receptor (TCR), all of which activate survival pathways alongside proapoptotic pathways. By coupling an apoptotic signaling pathway to receptors that are activated by immune stimuli (PAMPs, proinflammatory cytokines, and antigens), immune cells can control their life span and restrict the extent of their activation, which can be inherently self-damaging.

Although IKKβ-deficient fibroblasts, hepatocytes, and macrophages are extremely sensitive to either TLR4- or TNFR1-induced apoptosis (Hsu et al., 2004; Li et al., 1999), the mechanisms by which NF-κB counteracts apoptosis are stimulus and cell type specific. For instance, whereas PAI-2 is an important inhibitor of TLR4-induced macrophage apoptosis, we did not observe an antiapoptotic function for PAI-2 in TNFR1-triggered fibroblast apoptosis. Also, inhibition of p38α has no effect on TNFR1-triggered fibroblast apoptosis, but it has a huge effect on survival of TLR4-triggered macrophages. Furthermore, RelA/p65-deficient fibroblasts and hepatocytes are highly susceptible to TNFR1-induced apoptosis (Beg et al., 1995), but unlike IKKβ-deficient macrophages, RelA-deficient macrophages are not hypersensitive to TLR4-induced apoptosis (A.H., unpublished results). In all likelihood, the differential requirements for signal transducers and transcription factors in prevention of TNFR1- and TLR4-induced apoptosis reflect the mechanistic dissimilarity of the proapoptotic reactions that the two stimuli elicit.

Whereas the role of IKKβ-driven NF-κB activation in innate immunity has been well explored and is tightly linked to its antiapoptotic function (Karin and Lin, 2002), this work sheds new light on the role of p38 in macrophage function and innate immunity. By regulating CREB activity, through its subordinate kinases MSK1/2 and additional targets, p38 is an important regulator of macrophage survival that is required for induc-

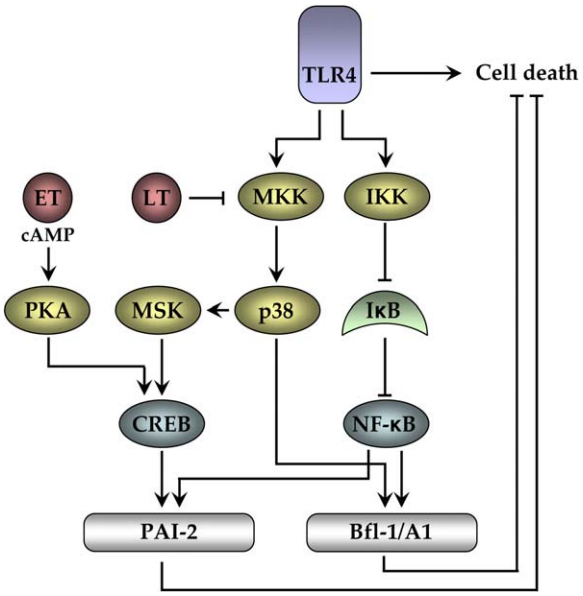


Figure 7. A Wiring Diagram Illustrating the Signaling Pathways through which TLR4 Activation Inhibits Macrophage Apoptosis

Engagement of TLR4 triggers prosurvival and prodeath pathways. The survival response depends on activation of IKK and p38 MAPK, which synergize to induce transcription of two major antiapoptotic genes, *Bfl-1/Al* and *Pai-2*. Induction of *Pai-2* depends on activation of two p38-subordinate kinases, MSK1/2, which phosphorylate and activate transcription factor CREB. The latter can also be accomplished through activation of PKA in response to cAMP, whose production is catalyzed by the EF subunit of anthrax ET. This response can delay the onset of macrophage death triggered by the action of LF, the catalytic subunit of anthrax LT, which prevents p38 activation by cleaving MKK6.

tion of two critical antiapoptotic genes: *Pai-2* and *Bfl-1/A1*. Unlike IKKβ and NF-κB, which regulate numerous proinflammatory and innate immune genes, the transcriptional function of p38 is more limited. Nonetheless, p38 activation is also of importance for other macrophage functions, such as phagocytosis (Blander and Medzhitov, 2004; Doyle et al., 2004), which does not depend on NF-κB activation (unpublished results). Although the direct targets for p38 in the pathway that leads to *Pai-2* induction are MSK1/2, which are required for CREB activation, its target in the pathway leading to *Bfl-1/A1* induction remains to be identified. Previous work has shown that some NF-κB target genes may depend on p38 for chromatin modification (Saccani et al., 2002), and the *Bfl-1/A1* locus may represent such a target.

CREB and its target genes participated in a plethora of biological processes such as glucose metabolism, neuroendocrine control, neuronal development, and synaptic plasticity (Mayr and Montminy, 2001). We have found that, acting as an antiapoptotic transcription factor in macrophages, CREB is also involved in innate immunity. In macrophages actively engaged in TLR4-mediated immunity, CREB promotes survival, at least in part, through induction of PAI-2. An antiapoptotic role for CREB has also been demonstrated in other cell types such as neurons (Bonni et al., 1999; Riccio et al., 1999; Lonze et al., 2002; Mantamadiotis et al., 2002), colon cancer cells (Nishihara et al., 2004), and pancreatic β-cells (Jhala et al., 2003). It is noteworthy that, unlike neurons and β-cells, where CREB activation alone drives expression of survival genes such as Bcl-2 and IRS2, respectively (Jhala et al., 2003; Lonze et al., 2002; Mantamadiotis et al., 2002), induction of *Pai-2* gene transcription in macrophages requires activation of both CREB and NF-κB. Conceivably, by linking the CREB-dependent antiapoptotic response to IKK/NF-κB activation, macrophages couple this response to innate immune stimuli.

The ability of anthrax ET to restore CREB activity when the activating signal from TLR4 is blocked by LT raises an interesting possibility regarding inhalation anthrax. *B. anthracis* interacts with macrophages in a paradoxical manner: it induces killing of alveolar macrophages to suppress innate immune responses, yet it utilizes these cells as a medium for replication and as a vehicle for spreading to regional lymph nodes en route to a fatal systemic infection (Dixon et al., 1999). While LF is responsible for inhibiting p38-dependent antiapoptotic signaling and inducing the death of TLR4-activated macrophages (Park et al., 2002), EF may be used to delay this apoptotic response until *B. anthracis* has replicated to a sufficiently high titer within the macrophage. Consistent with this notion, EF prevents inhibition of the CREB-dependent antiapoptotic response without inducing expression of other CREB-independent p38 and NF-κB target genes that are involved in innate immunity. This selective restoration of CREB-mediated survival could be important for keeping other LT-sensitive immune responses inactive during the course of *B. anthracis* infection.

Experimental Procedures

Mice and Macrophages

Ikkβ$^{-/-}$ (Li et al., 1999), *Ikkβ*$^{\Delta mye}$ (Greten et al., 2004), *Ikkα*$^{AA/AA}$ (Cao et al., 2001), and *p38α*$^{F/F}$ (Nishida et al., 2004) mice were described previously. *p38α*$^{\Delta mye}$ mice were generated by crossing *p38α*$^{F/F}$ mice with *LysM-Cre* mice (Clausen et al., 1999). BMDMs and FLDMs were prepared as described (Park et al., 2002). For infection experiments, the *B. anthracis* Sterne strain 7702 (Hsu et al., 2004) was grown and added to macrophage cultures as previously described (Park et al., 2004).

Reagents

Recombinant LF, EF, and PA were expressed in and purified from *E. coli* strain BL21 (DE3) bearing the appropriate plasmid constructs as described (Cunningham et al., 1998). LPS (*E. coli*) and forskolin were from Sigma-Aldrich, MAPK inhibitors were from Calbiochem, and cAMP analogs were from BIOLOG.

Measurement of Apoptosis

TUNEL assays and Hoechst staining were performed as described (Park et al., 2002). MTT assays and annexin V staining were performed using an MTT kit and annexin V-Alexa 568, respectively, according to manufacturer's instructions (Roche).

Protein and RNA Analysis

Whole-cell, cytoplasmic, and nuclear extracts for immunoblot analysis were prepared and analyzed as described (Park et al., 2004). Total RNA was isolated using RNAwiz (Ambion). Microarray analysis was performed with Affymetrix mouse chips with probes for more than 8000 genes and total RNAs from J774A.1 macrophages that had been unstimulated or stimulated with LPS for 4 hr. RNA analysis using real-time PCR was performed as described

(Park et al., 2004). Individual primer sequences are available upon request.

Chromatin Immunoprecipitation

ChIP assays were as described (Saccani and Natoli, 2002). Polyclonal antibodies to p65/RelA (C-20), c-Rel (C), and Pol II (N-19) were from Santa Cruz. The sequences of the promoter-specific primers (*Pai-2*, –1351 to –827 for NF-κB and CREB and –249 to –40 for Pol II; *IκBα*, –207 to –79; *iNos*, –261 to –72; *Bfl-1/A1*, –428 to –173) and a detailed experimental protocol are available upon request.

Lentiviral Vectors

To construct the lentiviral vectors for RNAi, oligonucleotides with the following sequences were synthesized, annealed, and cloned in pLentiLox3.7 as described (Rubinson et al., 2003): Bfl-1/A1, 5′-tggaaatgctctttctcctcaattcaagagattgaggagaaagagcatttccttttttc-3′ and 5′-tcgagaaaaaaggaaatgctctttctcctcaatctcttgaattgaggagaaagagcatttcca-3′; Luc, 5′-tgaaaattgtgtacctttagctttcaagagaagctaaaggtacacaattttctttttc-3′ and 5′-tcgagaaaaaagaaaattgtgtacctttagcttctcttgaaagctaaaggtacacaattttca-3′. GFP-, ACREB-, and shRNA-expressing lentiviruses were produced as described elsewhere (Rubinson et al., 2003). For lentiviral infection, BMDMs were incubated with viruses for 48 hr before LPS treatment.

Supplemental Data

Supplemental Data include eight figures and can be found with this article online at http://www.immunity.com/cgi/content/full/23/3/319/DC1/.

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