Cell PRESS
Immunity
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# Cyclic Adenosine Monophosphate Suppresses the Transcription of Proinflammatory Cytokines via the Phosphorylated c-Fos Protein

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DOI 10.1016/j.immuni.2008.12.021

## SUMMARY

**Intracellular cyclic adenosine monophosphate (cAMP) suppresses innate immunity by inhibiting proinflammatory cytokine production from monocytic cells. Enhanced expression of interleukin-10 (IL-10) has been suggested to be the mechanism of suppression. However, cAMP is still capable of suppressing production of the cytokines TNF-α and IL-12 in IL-10-deficient dendritic cells (DCs). Here, we demonstrated that the transcription factor c-Fos was responsible for the cAMP-mediated suppression of inflammatory cytokine production. c-Fos accumulated at high amounts in response to cAMP and lipopolysaccharide (LPS). Overexpression of c-Fos suppressed LPS-induced cytokine production, whereas cAMP-mediated suppression of TNF-α and IL-12 was impaired in *Fos*$^{-/-}$ DCs or in RAW264.7 cells treated with c-Fos siRNA. c-Fos physically interacted with p65 protein and reduced the recruitment of p65 to the *Tnf* promoter. Multiple sites of c-Fos were phosphorylated by the IKKβ protein. Thus, we propose that c-Fos is a substrate of IKKβ and is responsible for the immunosuppressive effect of cAMP.**

## INTRODUCTION

The Toll-like receptor (TLR) signaling pathway plays a central role in innate immunity and is linked to the activation of adaptive immunity. TLRs are activated by interaction with their cognate ligands, such as lipopolysaccharide (LPS), followed by the activation of two distinct adaptor molecules, MyD88 and TRIF. The MyD88 and the TRIF signaling pathways activate the transcription factors, NF-κB and IRF3, leading to the transcriptional activation of proinflammatory cytokines (TNF-α, IL-6, and IL-12) and interferons (IFNs) (Takeda and Akira, 2004). The TLR signal is strictly regulated by anti-inflammatory cytokines, such as IL-10 (Takeda et al., 1999), as well as various chemical and peptide mediators, including prostaglandin E2 (PGE2) (Harris et al., 2002; Kabashima et al., 2002; Shiraishi et al., 2008), histamine (Jutel et al., 2002), extracellular ATP (la Sala et al., 2001), vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating peptide (PACAP) in order to maintain homeostasis (Pozo, 2003). IL-10 has been shown to suppress proinflammatory cytokine production through the transcription factor STAT3 (Takeda et al., 1999), whereas other mediators act by elevating the concentration of intracellular cyclic adenosine monophosphate (cAMP).

cAMP has inhibitory effects on a variety of components of cell activation, including phagocytosis (Aronoff et al., 2004), reactive oxygen intermediate (ROI) generation (Dent et al., 1994), and the production of inflammatory mediators such as TNF-α (Rowe et al., 1997). The modulation of macrophage activation is important for immunoregulation as well as resolution of inflammation, yet the downstream mechanisms involved in these cAMP effects remain to be elucidated.

Protein kinase A (PKA) was earlier thought to be the primary effector of cAMP in eukaryotic cells (Walsh et al., 1968). In addition, exchange protein directly activated by cAMP (Epac) also functions as a receptor for cAMP (de Rooij et al., 1998; Kawasaki et al., 1998). Several reports suggested that PKA, rather than Epac, is important for cAMP-mediated suppression of cytokine production, whereas activation of Epac, but not PKA, dose dependently suppresses phagocytosis (Aronoff et al., 2005). However, the anti-inflammatory effect downstream of PKA or Epac has not been discovered.

Antigen-presenting cells (APCs) treated with cAMP-elevating reagents express reduced expression of costimulatory molecules and enhanced amounts of IL-10 (Akasaki et al., 2004; Delgado et al., 2005), which induce Tr1-type regulatory T cells. The upregulation of IL-10 by cAMP has been suggested to be an important mechanism of suppression. However, our present study demonstrated that cAMP was still capable of suppressing TNF-α and IL-12 production in IL-10-deficient dendritic cells (DCs). Therefore, an additional important mechanism other than IL-10 is considered to exist. By using microarray analysis, we identified c-Fos as a strong candidate for the mediator of cAMP because it has been reported that deletion of the *Fos* gene (encoding c-Fos) results in hyperinduction of proinflammatory cytokines in response to LPS (Ray et al., 2006). However, the mechanism of c-Fos-mediated suppression of proinflammatory cytokine production has not been elucidated. We found that the

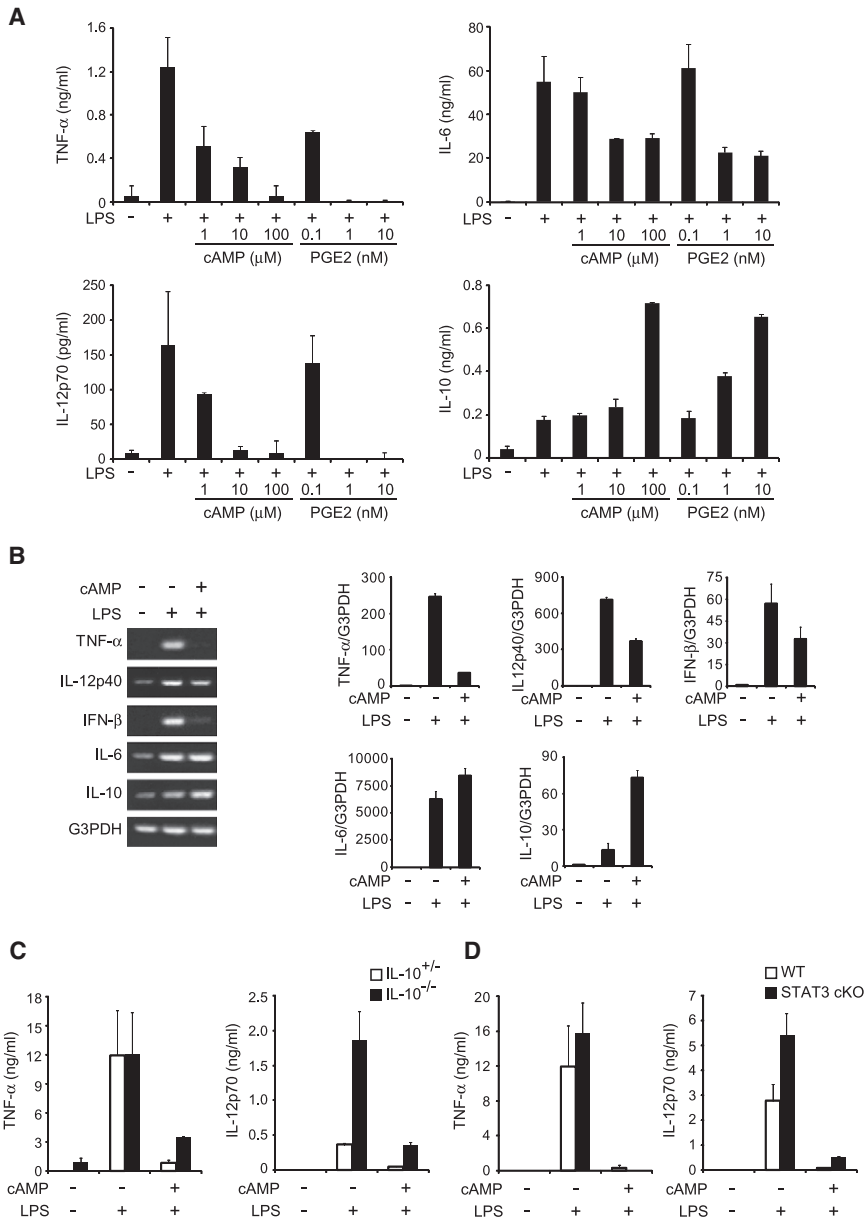


**Figure 1. Effect of 8-bromo-cAMP and PGE2 on LPS-Induced Cytokine Production in BMDCs**

(A) BMDCs were stimulated with LPS (10 ng/ml), cAMP, and PGE2 at the indicated concentrations for 24 hr. The concentration of TNF-α, IL-6, IL-10, and IL-12p70 in the culture supernatants was measured by ELISA.

(B) BMDCs were stimulated with LPS in the presence or absence of cAMP (100 μM) for 3 hr, and the mRNA was analyzed by RT-PCR (left panel) and real-time PCR (right panel).

(C and D) BMDCs from (C) *Il10*$^{+/-}$ mice or *Il10*$^{-/-}$ mice or those from (D) wild-type mice or Tie2-Cre-*Stat3*$^{fl/fl}$ mice were stimulated with LPS and cAMP for 24 hr. The concentrations of cytokines were measured by ELISA.

All graphs are mean ± SD. n = 2. Representative of three experiments.

amounts of c-Fos protein induced by LPS+cAMP were much higher than those induced by LPS or cAMP alone. cAMP mostly upregulated c-Fos mRNA expression, whereas the kinase IKKβ activated by LPS directly phosphorylated c-Fos, leading to c-Fos protein stabilization and accumulation. We identified one of the critical residues of c-Fos for IKKβ-mediated phosphorylation and stabilization. These data indicate that c-Fos is a substrate of IKKβ and a therapeutic target for the suppression of endotoxin shock and inflammatory diseases.

## RESULTS

### cAMP Suppresses TNF-α and IL-12 Production Independently of IL-10

To investigate the molecular mechanism by which cAMP suppresses proinflammatory cytokine production, we first confirmed that cAMP-activating reagents (PGE2), as well as the membrane-permeable cAMP analog 8-bromo-cAMP (simply referred to as cAMP hereafter in this paper), equally suppressed TLR-mediated proinflammatory cytokine production. As reported previously (Katakami et al., 1988; Kunkel et al., 1988), PGE2 and cAMP strongly suppressed LPS-induced TNF-α and IL-12p70 production from bone marrow-derived dendritic cells (BMDCs) (Figure 1A) as well as from RAW264.7 cells (data not shown). IL-6 protein was decreased by only half at the maximum amount of cAMP or PGE2. cAMP suppressed the mRNA expression of TNF-α, IL-12p40, and IFN-β, but not those of IL-6 induced by LPS in BMDCs (Figure 1B). In contrast, the amount of anti-inflammatory cytokine IL-10 was elevated by LPS in the presence of cAMP or PGE2 in BMDCs (Figures 1A and 1B).

IL-10 is known to suppress the production of proinflammatory cytokines through STAT3 (Takeda et al., 1999). Figures 1C and 1D show that cAMP still suppressed TNF-α and IL-12p70 production in BMDCs from IL-10- or STAT3-deficient mice. These results indicate that a mechanism independent of the IL-10-STAT3 pathway exists for the suppression of proinflammatory cytokine production by cAMP.

### cAMP Does Not Affect Intracellular TLR Signaling

Next, we examined the effect of cAMP on LPS signaling. LPS induces the production of proinflammatory cytokines through intracellular phosphorylation signaling cascades activated by TLR4. As shown in Figure S1A available online, the phosphorylation and degradation of IκBα, as well as the phosphorylation of MAP kinases, ERK, p38, and JNK, were not affected by cAMP. Upon TLR signaling, the transcription factors NF-κB and IRF3

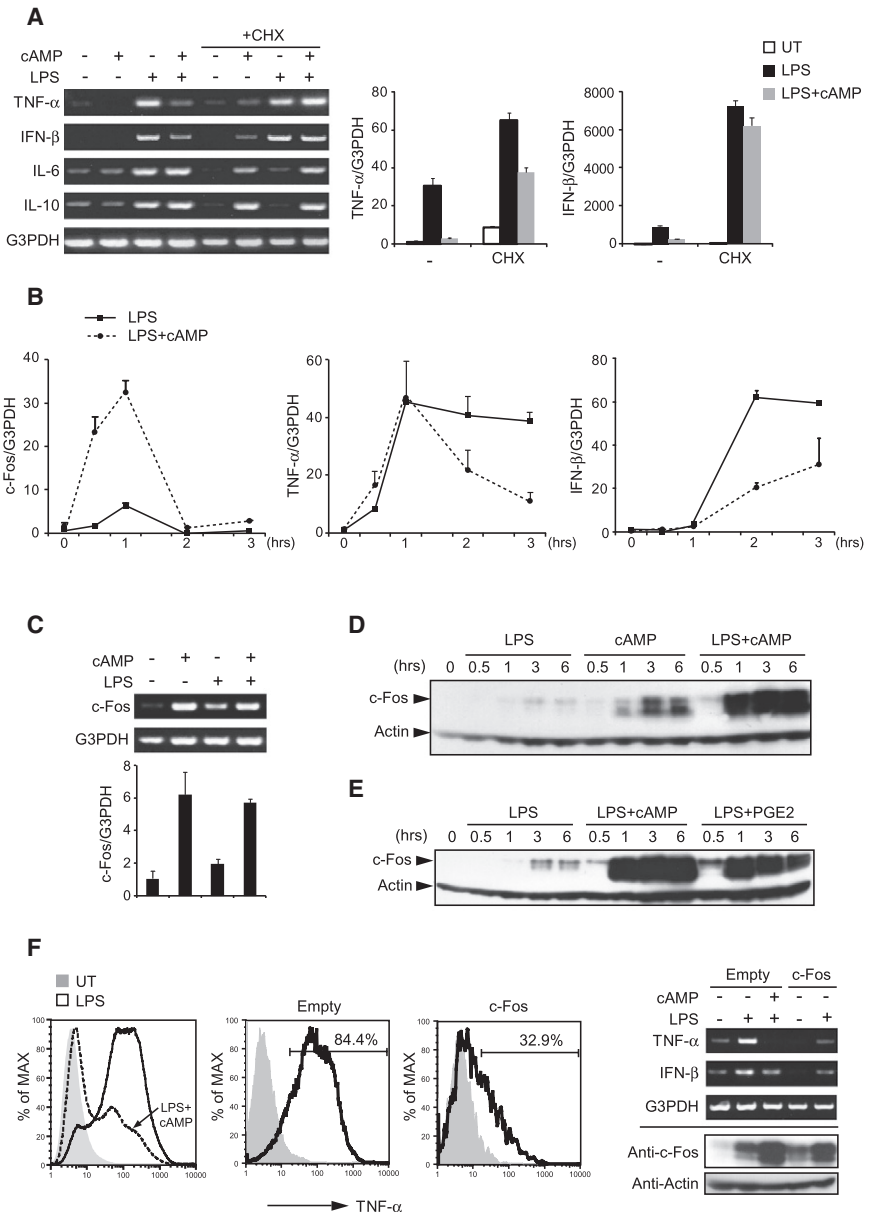


**Figure 2. c-Fos Protein Accumulation by LPS+cAMP and c-Fos Overexpression Suppresses TNF-α Production**

(A) RAW264.7 cells were treated with cycloheximide (CHX; 20 μg/ml) and then stimulated with LPS (10 ng/ml) and cAMP (100 μM) for 3 hr. The mRNA expression was analyzed by RT-PCR and real-time PCR. The gels show expression of the indicated mRNA, and the graphs show quantitation of TNF-α and IFN-β expression as a ratio of G3PDH.

(B) Time course of the induction of c-Fos mRNA by cAMP+LPS. RAW264.7 cells were stimulated with LPS and cAMP for the indicated time. The c-Fos, TNF-α, and IFN-β mRNA were analyzed by real-time PCR.

(C) Induction of c-Fos mRNA by cAMP and LPS. RAW264.7 cells were stimulated with cAMP and LPS for 1 hr, and the c-Fos mRNA was analyzed by RT-PCR and real-time PCR.

(D and E) Time course of the induction of c-Fos protein by (D) LPS+cAMP or (E) LPS+PGE2. RAW264.7 cells were stimulated with LPS, cAMP, and PGE2 (10 nM) for the indicated time. The c-Fos protein was analyzed by immunoblot.

(F) RAW264.7 cells were transfected with empty EGFP or c-Fos-IRES-EGFP vectors. After 24 hr, cells were stimulated with LPS in the presence of Brefeldin A for 6 hr, followed by intracellular staining by the TNF-α antibody. TNF-α expression in GFP-positive cells was analyzed by FACS (left panel). GFP-positive cells were sorted by FACS and then stimulated with LPS in the presence or absence of cAMP for 3 hr. The mRNA of indicated genes was analyzed by RT-PCR, and c-Fos proteins were analyzed by immunoblot (right panel).

All graphs are mean ± SD. n = 2. Representative of three experiments.

were translocated into the nucleus and upregulated cytokine mRNA transcription. The nuclear localization of the p65 subunit of NF-κB and IRF3 was also unaffected by cAMP (Figure S1B). These results indicate that the suppression by cAMP is not due to the inhibition of the LPS-mediated intracellular signal transduction pathways.

## c-Fos Is a Candidate for the Mediator of the Inhibitory Effect of cAMP

We next determined whether the suppression of cytokine mRNA by cAMP requires de novo protein synthesis. In RAW264.7 cells, the protein synthesis inhibitor cycloheximide (CHX) inhibited the suppressive effect of cAMP on TNF-α and IFN-β mRNA (Figure 2A). Therefore, a protein induced by the LPS+cAMP signals likely suppressed proinflammatory cytokine mRNA synthesis induced by LPS. A microarray analysis was used to identify the genes induced by LPS+cAMP, but not by LPS, stimulation. One of the genes induced by LPS+cAMP, but not by LPS alone, was inducible cAMP early repressor (ICER), which has been shown to be involved in the suppression of TNF-α production by Calcitonin gene-related peptide (CGRP) (Harzenetter et al., 2007). However, no suppression of LPS-induced TNF-α production by *Crem* (encoding ICER) overexpression in RAW264.7 cells was observed in our system (data not shown). Thus, we searched other targets and found that c-Fos, a member of the AP-1 transcription factor, was upregulated by cAMP+LPS. The c-Fos mRNA expression increased much more strongly by LPS+cAMP than by LPS alone (Figure 2B). We decided to analyze c-Fos further because c-Fos mRNA was upregulated prior to the decrease of the TNF-α and IFN-β mRNA expression by cAMP (Figure 2B). In addition, c-Fos has been reported to negatively regulate LPS-mediated cytokine production in macrophages (Ray et al., 2006).

cAMP signals seem to be important for the induction of c-Fos mRNA because no additive effect was observed when cAMP was given with LPS (Figure 2C). We also observed a marked

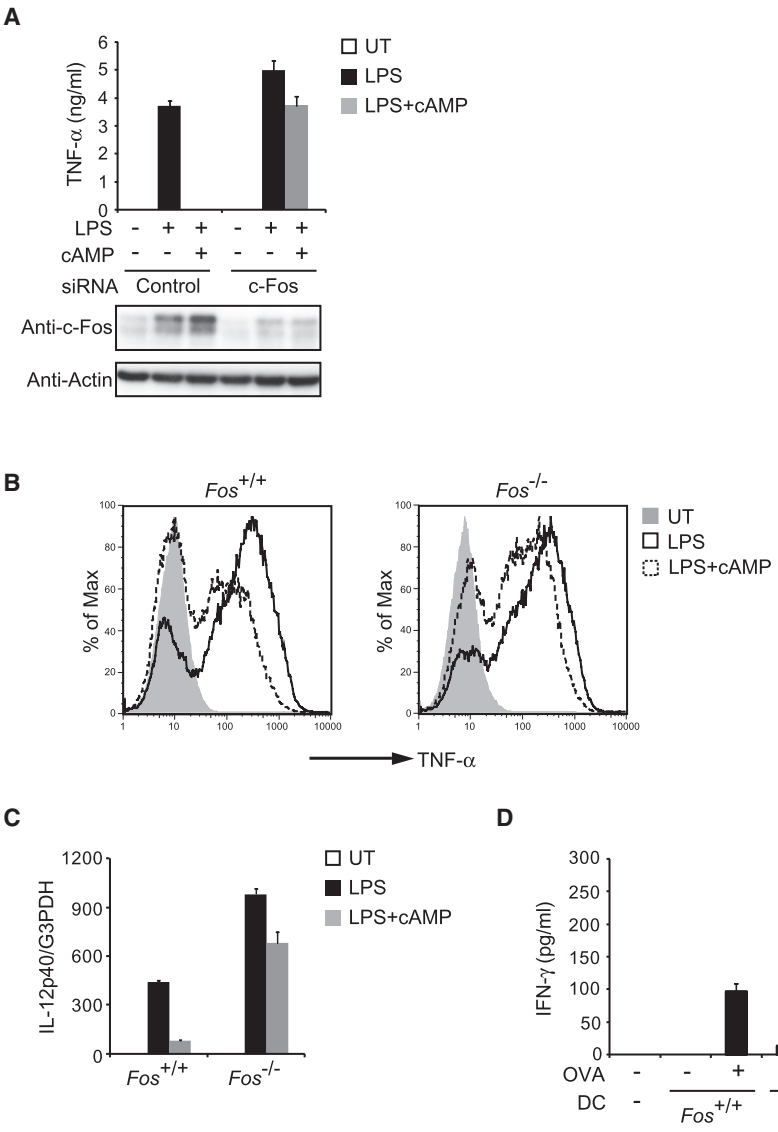


**Figure 3. c-Fos Is Required for cAMP-Mediated Suppression of Cytokine Production**

(A) The effect of *Fos* knockdown by RNAi. c-Fos siRNA was transfected into RAW264.7 cells. After 24 hr, cells were stimulated with LPS (10 ng/ml) and cAMP (100 μM) for 3 hr. The concentrations of TNF-α were measured by ELISA (upper panel), and c-Fos protein was analyzed by immunoblot (lower panel).

(B) BMDCs from LysM-Cre-*Fos*$^{fl/fl}$ (indicated as *Fos*$^{-/-}$) and WT (*Fos*$^{+/+}$) mice were stimulated with LPS in the presence of Brefeldin A for 6 hr, followed by intracellular staining by the TNF-α antibody.

(C) IL-12p40 mRNA expression in BMDCs from LysM-Cre-*Fos*$^{fl/fl}$ (*Fos*$^{-/-}$) mice were analyzed by real-time PCR.

(D) The effect of c-Fos deficiency in DCs in vivo. *Fos*$^{+/+}$or *Fos*$^{-/-}$mice spleen-derived DCs were injected into OT-II mice. After 7 days, spleen and lymph node cells were isolated and cultured with PMA and ionomycin for 6 hr. The concentrations of IFN-γ were measured by ELISA.

All graphs are mean ± SD. n = 3. Representative of two experiments.

increase of the c-Fos protein in response to LPS+cAMP (Figure 2D) or LPS+PGE2 (Figure 2E). Although c-Fos mRNA induction was transient and decreased after 1 hr stimulation with LPS+cAMP (Figure 2B), the amount of c-Fos protein was mainteined even after 6 hr treatment (Figures 2D and 2E). The increase in c-Fos protein by LPS+cAMP was also observed in BMDCs (data not shown). These data suggest that high accumulation of c-Fos protein in response to cAMP+LPS might affect LPS-induced inflammatory cytokine induction.

## Forced Expression of c-Fos Inhibits TNF-α Production

To determine whether elevation of c-Fos protein was sufficient for the suppression of proinflammatory cytokine production, we measured TNF-α protein and mRNA expression in RAW264.7 cells by intracellular immunostaining followed by flow cytometry and RT-PCR analysis, respectively. RAW264.7 cells were transfected with *Fos* cDNA linked to the IRES-EGFP or an empty vector carrying EGFP. After LPS stimulation, the TNF-α expression was analyzed in GFP-positive cell fractions. As shown in Figure 2F (left panel), transduction of *Fos* cDNA suppressed TNF-α protein induction. This was confirmed by RT-PCR. GFP-positive cells were sorted, and then TNF-α mRNA expression was examined by RT-PCR. LPS-induced TNF-α mRNA induction was markedly inhibited by *Fos* transduction (Figure 2F, right panel). Similarly, LPS-induced IFN-β mRNA expression was reduced by *Fos* overexpression (Figure 2F, right panel). c-Fos protein expressed by cDNA transfection after stimulation with LPS alone was almost comparable to c-Fos expression in cells treated with cAMP+LPS (Figure 2F, right panel), suggesting that the elevated c-Fos protein expression induced by cAMP+LPS was high enough to suppress TNF-α production.

## c-Fos Is Essential for cAMP-Dependent Suppression of Inflammatory Cytokine Production

Next, to investigate whether c-Fos is necessary for cAMP-mediated suppression of TNF-α production, we performed *Fos* RNAi analysis in RAW264.7 cells. When c-Fos protein expression was reduced by siRNA, cAMP-mediated suppression of TNF-α production was hardly observed (Figure 3A). In addition, overexpression of the dominant-negative c-Fos mutant A-Fos (Olive et al., 1997) cancelled the suppressive effect of cAMP on LPS-induced TNF-α and IFN-β mRNA upregulation (Figure S2). These data indicate that c-Fos is necessary for the suppressive effect of cAMP in RAW264.7 cells.

We further examined the physiological significance of c-Fos in the cAMP-mediated suppression of inflammatory cytokine production with *Fos*-disrupted cells. Dendritic cells (DCs) and

macrophages from *Fos*-deficient mice were analyzed for LPS-induced TNF-α mRNA and protein expression (Figures 3B, 3C, and S3). Suppression of TNF-α protein accumulation by cAMP was weaker in $Fos^{-/-}$ DCs than it was in $Fos^{+/+}$ DCs (Figure 3B). In fetal liver macrophages from $Fos^{-/-}$ mice, the suppressive effect of cAMP on the TNF-α mRNA expression was not as strong as it was in $Fos^{+/+}$ cells (Figure S3A). Similarly, the suppressive effect of cAMP on IL-12p40 mRNA expression was severely impaired in $Fos^{-/-}$ DCs and fetal liver macrophages (Figures 3C and S3A). These data indicate that c-Fos plays a critical role in the cAMP-mediated suppression of proinflammatory cytokine production.

To see the effect of *Fos* deficiency on DCs in vivo, we adoptively transferred *Fos*-deficient DCs into TCR-Tg (OT-II) mice after antigen uptake. As shown in Figure 3D, the production of IFN-γ from $Fos^{-/-}$ DC-transferred mice was higher than that of $Fos^{+/+}$ DC-transferred mice. These results suggest that c-Fos functions as an anti-inflammatory mediator in vivo.

## Recruitment of p65 to the Tnf Promoter Is Blocked by c-Fos

The *Tnf* promoter was analyzed to elucidate the mechanism of suppression by c-Fos. The −2.0 kb promoter region was cloned and fused to the luciferase gene. LPS strongly upregulated this *Tnf* promoter activity, which was suppressed by cAMP (Figure 4A). Deletion analysis of the promoter region revealed that −700 to −650 bp of the *Tnf* promoter containing the NF-κB binding site (−670 κB site) was important for LPS-induced transcriptional activation as well as suppression by cAMP. The ChIP assay confirmed that the p65 subunit of NF-κB can interact with this region (−700 to −490 bp) in response to LPS and that the recruitment of p65 was blocked by cAMP (Figure 4B, left, upper panels). In contrast, p65 recruitment was not reduced but, rather, was enhanced by cAMP in the *Il6* promoter region (Figure 4B, left, lower panels). c-Fos and c-Jun were also recruited to this region in a cAMP-independent manner (Figure 4B, right panel).

To investigate whether c-Fos also blocks p65 recruitment to the promoter, we transfected *Traf6* and *Fos* cDNA into RAW264.7 cells. TRAF6 is a mediator of TLR signaling, which activates the IKK complex and NF-κB. Overexpression of *Traf6* activated the *Tnf* promoter, but coexpression of *Fos* suppressed the promoter activation (Figure 4C, left panel). Under these conditions, *Traf6* enhanced recruitment of p65 to the promoter, whereas *Fos* overexpression suppressed this recruitment (Figure 4C, right panel). These data suggest that both cAMP and c-Fos suppress the induction of TNF-α mRNA by blocking p65 recruitment to its promoter region.

To understand how c-Fos inhibits p65 recruitment to the promoter region, we also performed DNA affinity precipitation (DNAP) assays with the oligonucleotide of the *Tnf* promoter region (−670 κB site). In LPS-stimulated RAW264.7 cell nuclear extracts, p65, but not p50, was bound to this oligonucleotide (Figure 4D). Lack of binding of p50 to the −670 κB site was confirmed in nuclear extracts from HEK293T cells overexpressing p65 and p50 (Figure S4). These data suggest that the −670 κB site is preferentially recognized by the p65:p65 homodimer, whereas the NF-κB consensus sequence is recognized by the p50:p65 heterodimer, which is consistent with previous reports (Bohuslav et al., 1998; Chen et al., 1998). The amount of p65 bound to the −670 κB site oligonucleotide in the cell extracts stimulated with LPS+cAMP was reduced compared with those stimulated with LPS alone (Figure 4D). However, binding of p65 to the NF-κB consensus oligonucleotide was not inhibited by cAMP (Figure 4D). Similar reduction of p65 binding to the −670 κB site oligonucleotide was observed when the nuclear extracts were incubated with recombinant GST-c-Fos protein (Figure 4E). As shown previously (Stein et al., 1993), p65 directly bound to c-Fos (full-length 1–380 aa) (Figure 4F). The deletion mutant c-Fos (111–380), but not c-Fos (1–220), interacted with p65 (Figure 4F). c-Fos (111–380) as well as WT-c-Fos (1–380), but not c-Fos(1–220), inhibited p65-mediated *Tnf* promoter luciferase activation (Figure 4G, left panel). c-Fos (111–380), but not c-Fos (1–220), blocked binding of p65 to the −670 κB site oligonucleotide (data not shown). In contrast, *Fos* overexpression did not affect p65-induced NF-κB consensus-promoter activation (Figure 4G, right panel). Taken together, direct binding of c-Fos to p65 seems to reduce affinity of p65 to the −670 κB site, but not to the consensus κB site. A schematic model for the c-Fos-mediated *Tnf* promoter suppression is shown in Figure S5.

## cAMP Upregulates c-Fos mRNA, whereas LPS Stabilizes c-Fos Protein

We then investigated the molecular mechanism by which c-Fos protein was drastically accumulated by LPS+cAMP. The c-Fos protein has been reported to be very unstable and rapidly degraded (Stancovski et al., 1995; Tsurumi et al., 1995). Phosphorylation by several kinases plays important roles in both the degradation and stabilization of c-Fos protein (Chen et al., 1993; Coronella-Wood et al., 2004; Murphy et al., 2002; Okazaki and Sagata, 1995; Sasaki et al., 2006; Tanos et al., 2005). Therefore, we tested the effect of inhibitors of protein kinases (Figures 5A and S6A). Suppression of activation of the kinase by each inhibitor was confirmed by immunoblot with an antibody specific to the phosphorylated (activated) form of the kinases (data not shown). Although the PKA inhibitor H89 partially suppressed c-Fos protein accumulation, other inhibitors of GSK-3β, ERK, p38, and JNK did not affect the c-Fos protein expression (Figure S6A).

Wortmannin, a PI3 kinase inhibitor, partly suppressed the c-Fos protein accumulation and c-Fos mRNA induction (Figure 5A). However, another PI3 kinase inhibitor, LY294002, did not affect c-Fos protein and mRNA accumulation (Figure 5A). Therefore, a wortmannin-sensitive protein kinase that is different from PI3 kinase or downstream kinases may be involved in the induction of c-Fos mRNA. Consistent with c-Fos protein expression, LY294002 did not affect cAMP-mediated suppression of TNF-α mRNA expression, whereas wortmannin partially reversed the effect of cAMP (Figure S6B). Interestingly, IKK-2 (IKKβ) inhibitor (IKK2 inhibitor IV) almost completely suppressed c-Fos protein accumulation but had little effect on c-Fos mRNA induction (Figure 5A). Requirement of IKKβ was confirmed by the reduction of IKKβ expression by siRNA (Figure 5B). IKKβ knockdown resulted in less c-Fos accumulation by LPS+cAMP stimulation. These data suggest that both LPS- and cAMP-induced signals are necessary for c-Fos protein accumulation; however, cAMP signals are important for c-Fos mRNA induction, whereas LPS-IKKβ signals are more important for c-Fos protein accumulation.

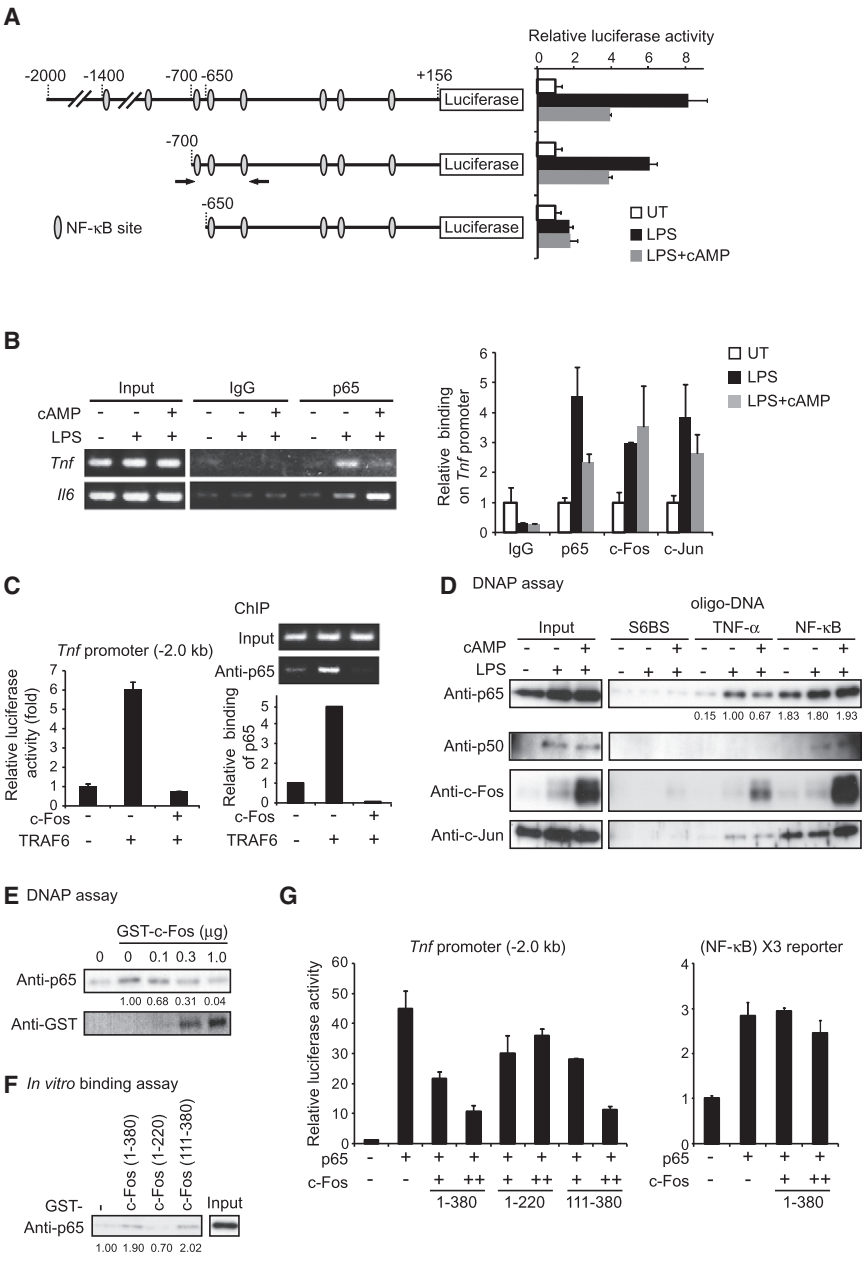


**Figure 4. The Effect of c-Fos on p65-Mediated *Tnf* Promoter Activation**

(A) RAW264.7 cells were transfected with the indicated constructs of the *Tnf* promoter (−2.0 kb, −0.7 kb, and −0.65 kb) luciferase. After 24 hr, cells were stimulated with LPS and cAMP for 3 hr and then luciferase activity was measured. The putative NF-κB sites were predicted by TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html), and the sites with scores higher than 70 are shown. Arrows indicate the positions of primers used for the ChIP assay.

(B) ChIP assay. RAW264.7 cells were stimulated with LPS and cAMP for 3 hr. DNA coprecipitated with indicated antibodies was amplified by PCR with primer sets for the *Tnf* promoter (−700 to −490 bp) and *Il6* promoter (−307 to −72 bp) regions (left panel). DNA coprecipitated with indicated antibodies was analyzed by real-time PCR with primer sets for the *Tnf* promoter (−800 to −600 bp) region (right panel).

(C) RAW264.7 cells were transfected with *Tnf* promoter (−2.0 kb) luciferase, TRAF6, and c-Fos expression vectors. After 24 hr, luciferase activities were measured (left panel). RAW264.7 cells were transfected with TRAF6 and c-Fos expression vectors, and 24 hr later, the ChIP assay was performed with primer sets for the *Tnf* promoter (−700 to −490 bp).

(D) Oligo-DNA precipitation (DNAP) assay. RAW264.7 cells were stimulated with LPS and cAMP for 3 hr. Nuclear extracts were incubated with biotinylated oligonucleotides corresponding to the STAT6-binding site (S6BS) (a negative control), −670 κB site of the *Tnf* promoter sequence (TNF-α), or NF-κB consensus sequence (NF-κB). Proteins precipitated with avidin beads were analyzed by immunoblotting with indicated antibodies.

(E) DNAP assay with −670 κB site *Tnf* promoter. Nuclear extracts of RAW264.7 cells stimulated with LPS in the presence of the indicated amount of recombinant GST-c-Fos protein were incubated with biotinylated oligonucleotides corresponding to the −670 κB site. The relative densities of oligonucleotide-associated p65 are indicated in the lower panel.

(F) Interaction between c-Fos and p65 in vitro. RAW264.7 cells were stimulated with LPS for 1 hr. The nuclear fraction was extracted and precipitated with recombinant GST-c-Fos full-length (1–380 aa) or deletion mutants (1–220 aa and 111–380 aa). The precipitates were blotted with p65 antibody.

(G) The effect of c-Fos on *Tnf* promoter activity. RAW264.7 cells were transfected with the *Tnf* promoter (−2.0 kb) luciferase (left) or NF-κB consensus (× 3) (right) luciferase together with p65 and c-Fos. After 24 hr, luciferase activity was measured.

All graphs are mean ± SD. n = 2. Representative of three (A–C) or two (D–G) experiments.

## IKKβ Directly Phosphorylates and Stabilizes c-Fos Protein

To investigate the molecular mechanism of the accumulation of c-Fos protein by LPS-IKKβ signaling, we examined the phosphorylation of c-Fos protein in RAW264.7 cells. In several experiments, c-Fos protein was detected as multiple bands on SDS-PAGE with different molecular weights. This is probably due to multiple phosphorylation because higher-molecular weight species were not present with phosphatase (CIAP) treatment (Figure 5C). We then examined the stability of c-Fos protein. c-Fos accumulated in response to pretreatment of RAW264.7 cells with cAMP+LPS, and then new protein synthesis was blocked by adding cycloheximide (CHX). CHX treatment resulted in a rapid disappearance of c-Fos protein; however, the upper band stayed much longer than the lower band (Figure 5D). Furthermore, both higher- and lower-molecular weight species of c-Fos protein were accumulated in the presence of the proteasome inhibitor MG132 in cAMP-treated RAW264.7 cells (Figure S7). These data suggest that phosphorylation of c-Fos protein by LPS signals blocked proteasomal degradation of c-Fos.

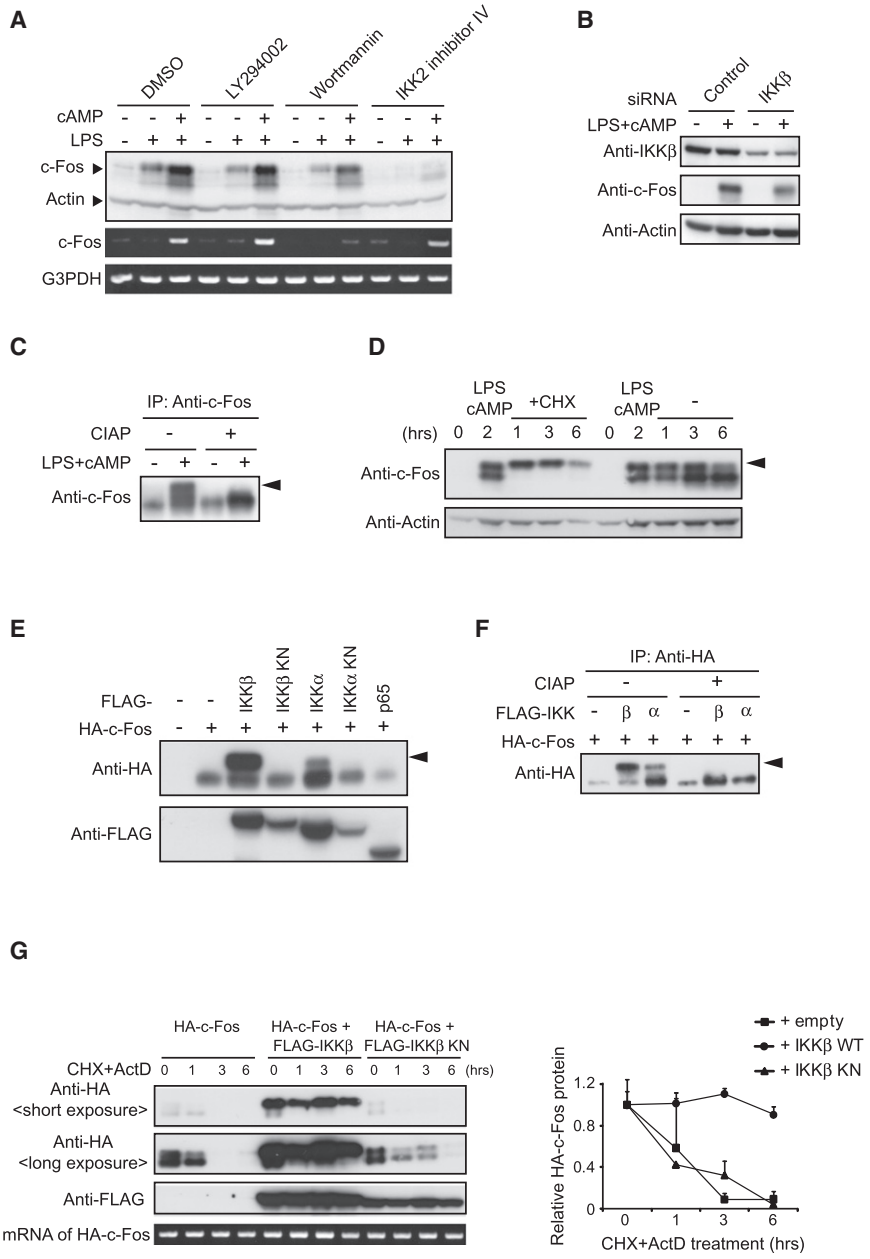


**Figure 5. IKKβ Phosphorylates and Stabilizes c-Fos Proteins**

(A) Effects of various kinase inhibitors on LPS+cAMP-induced c-Fos protein. RAW264.7 cells were pretreated with the inhibitors LY294002 (PI3K: 50 μM), wortmannin (PI3K: 100 nM), and IKK2 inhibitor IV (10 μM) for 30 min and stimulated with LPS and cAMP for 3 hr. The c-Fos protein and c-Fos mRNA were analyzed by immunoblot and RT-PCR, respectively.

(B) The effect of IKKβ knockdown on LPS+cAMP-induced c-Fos protein. IKKβ siRNA was transfected into RAW264.7 cells. After 24 hr, cells were stimulated with LPS (10 ng/ml) and cAMP (100 μM) for 3 hr. The IKKβ and c-Fos protein were analyzed by immunoblot.

(C) RAW264.7 cells were stimulated with LPS+cAMP, and cell extracts were immunoprecipitated by c-Fos antibody. Immunoprecipitates were treated by CIAP and analyzed by immunoblot.

(D) RAW264.7 cells were stimulated with LPS+cAMP for 2 hr and then treated with CHX (10 μg/ml) for the indicated periods. c-Fos protein was analyzed by immunoblot.

(E) FLAG-IKKβ, IKKβ KN (kinase-negative), IKKα, IKKα KN, or p65 was coexpressed with HA-c-Fos in HEK293T cells, and whole-cell extracts were analyzed by immunoblot.

(F) FLAG-IKKβ or IKKα were coexpressed with HA-c-Fos in HEK293T cells, and cell extracts were immunoprecipitated by HA antibody. Immunoprecipitates were treated by CIAP and analyzed by immunoblot.

(G) HA-c-Fos was expressed alone or with FLAG-IKKβ or FLAG-IKKβ KN in HEK293T cells, and 24 hr after transfection, cells were treated with CHX (10 μg/ml) and 1 μM of actinomycin D (ActD) for indicated periods. The protein and the mRNA of HA-c-Fos were analyzed by immunoblot and RT-PCR, respectively. Quantified relative HA-c-Fos protein amounts are shown below. Arrowheads indicate the lower-molecular weight species of c-Fos protein. All graphs are mean ± SD. n = 2. Representative of three experiments.

To examine whether IKKβ can directly induce phosphorylation and accumulation of c-Fos protein, we analyzed the c-Fos protein expression when IKKβ was coexpressed in HEK293T cells. As shown in Figure 5E, coexpression of IKKβ, but not a kinase-negative mutant of IKKβ (IKKβ KN), resulted in a drastic increase in the c-Fos protein. In addition, IKKβ expression reduced the mobility of c-Fos protein on SDS-PAGE. This reduced mobility of c-Fos was abolished by phosphatase treatment (Figure 5F), indicating that IKKβ phosphorylates c-Fos. IKKα also induced a similar mobility shift of c-Fos protein; however, generation of the lower-mobility species was much weaker compared with IKKβ (Figure 5E). An overexpression of active forms of MEKK1, c-Raf and Akt, neither enhanced the expression of c-Fos nor affected mobility on SDS-PAGE (data not shown). c-Fos protein expressed in HEK293T cells was degraded rapidly; however, the coexpression of IKKβ, but not IKKβ KN, prevented c-Fos protein from being degraded (Figure 5G). These data suggest that the phosphorylation by IKKβ resulted in the stabilization of c-Fos protein.

## Ser308 of c-Fos Is a Major Phosphorylation Site for IKKβ

Next, an in vitro kinase assay with GST-fused c-Fos proteins as substrates was used to investigate direct phosphorylation of c-Fos protein by IKKβ. GST-c-Fos (full 1–380), but not GST, was phosphorylated by IKKβ, but not by IKKβ-KN, as efficiently as the control IκBα (Figure 6A). IKKα was also able to phosphorylate GST-c-Fos, but the incorporation of $^{32}P$ into the substrate was much lower than it was by IKKβ (data not shown). This is

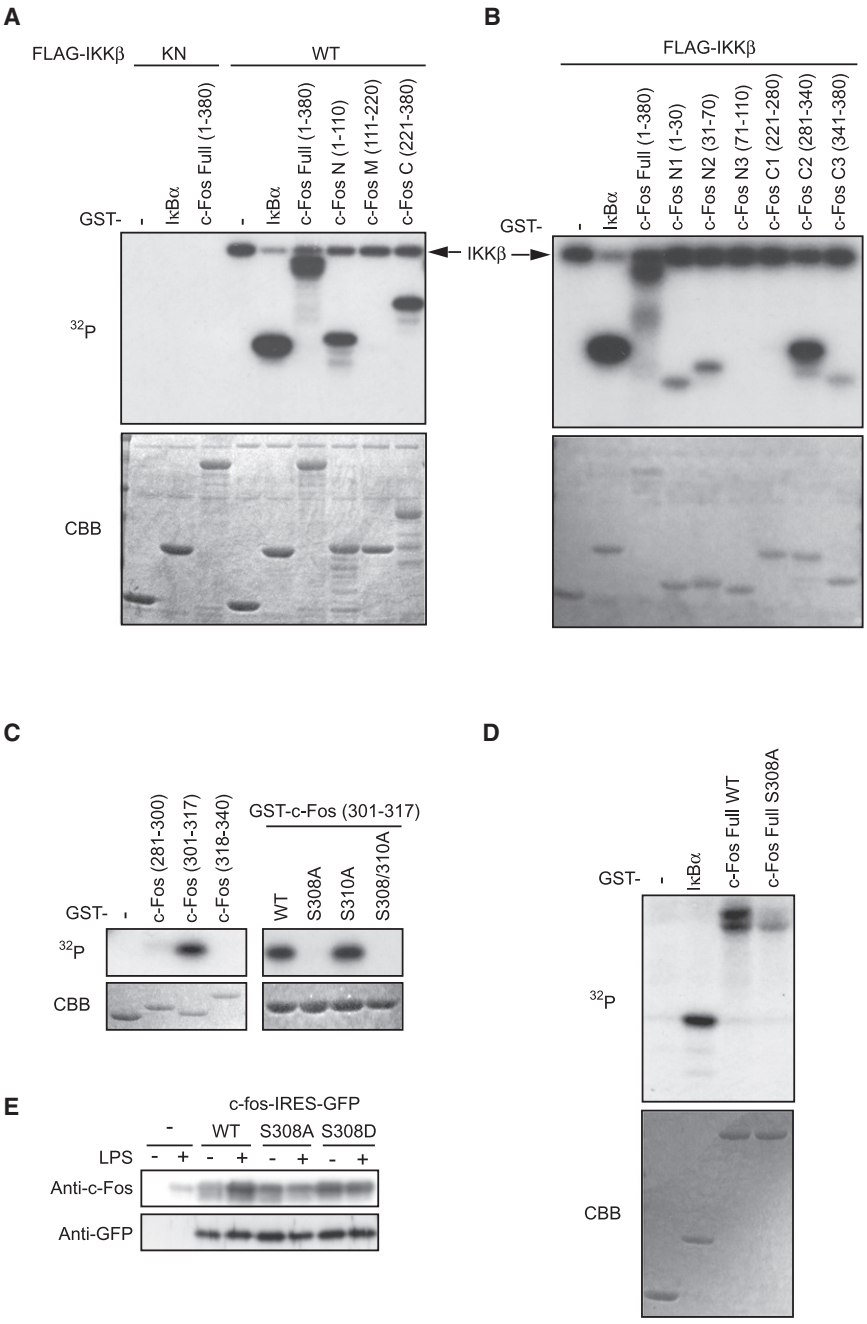


**Figure 6. S308 of c-Fos Is a Major Site of Phosphorylation by IKKβ and Is Required for Stabilization**

(A–D) In vitro kinase assays were performed by (A–C) FLAG-IKKβ or FLAG-IKKβ KN or by (D) LPS-activated endogenous IKK complex by using recombinant GST-c-Fos protein as a substrate. Phosphorylated proteins and total GST proteins were detected by autoradiography and CBB staining, respectively.

(E) c-Fos (WT), c-Fos (S308A), or c-Fos (S308D) constructs fused to IRES-EGFP were transfected into RAW264.7 cells, and 24 hr after transfection, cells were stimulated by LPS for 3 hr.

The whole-cell extracts were analyzed by immunoblot. Arrowheads indicate the lower-molecular weight species of c-Fos protein. Representative of three experiments.

consistent with weaker induction of slower-mobility c-Fos proteins by IKKα in HEK293T cells (Figure 5E). These data suggest that c-Fos is a more suitable substrate for IKKβ than for IKKα.

To define the sites of phosphorylation by IKKβ, we divided c-Fos into several regions. Both the N-terminal (1–110 aa) and C-terminal (221–380 aa) regions of c-Fos proteins were phosphorylated by IKKβ in vitro (Figure 6A). Then, N- and C-terminal regions were further divided into smaller regions with 30 to 60 amino acids. Multiple sites of c-Fos were apparently phosphorylated by IKKβ; however, region C2 (281–340) was most extensively phosphorylated (Figure 6B). None of the serine-threonine residues in this region have been recognized as phosphorylation sites by ERKs, RSK, or Mos (Chen et al., 1993; Murphy et al., 2002; Okazaki and Sagata, 1995; Sasaki et al., 2006). Thus, the C-terminal region was further narrowed by a series of deletions. This experiment determined that region 301–317 contained major phosphorylation residues in the C2 (218–340) region (Figure 6C, left). This region (301–317) contained only two serine residues, S308 and S310. We identified S308 as a major phosphorylation site by IKKβ in vitro (Figure 6C). To confirm the importance of this residue, we generated a full-length c-Fos mutant carrying an S308A substitution. GST-S308A c-Fos was very weakly phosphorylated by the endogenous IKK complex from LPS-stimulated RAW264.7 cells compared to WT c-Fos (Figure 6D). Thus, we concluded that S308 is one of the major sites of phosphorylation by IKKβ.

To investigate the role of S308 phosphorylation, we introduced S308A and S308D mutations into the *Fos* cDNA. S308D substitution may mimic the phosphorylation of c-Fos by IKKβ. When transiently expressed in RAW264.7 cells, the protein expression of WT c-Fos was upregulated by LPS stimulation, whereas that of c-Fos (S308A) was not upregulated by LPS (Figure 6E). Importantly, the expression of the S308D mutant was already high before LPS stimulation and was not further upregulated by LPS (Figure 6E). Similar data were obtained when these mutants were expressed in HEK293T cells together with IKKβ, although the expression of c-Fos (S308A) was still weakly upregulated by IKKβ (Figure S8). Nevertheless, these data suggest that S308 is one of the, if not the only, important residues for stabilization of c-Fos induced by phosphorylation with IKKβ.

## DISCUSSION

Stimulation with prostaglandin E2 (PGE2), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), histamine, and extracellular ATP has been reported to have an immunosuppressive effect on various cell types, including macrophages, DCs, and T cells. This suppressive effect is explained by the elevation of intracellular cAMP through the activation of adenyl cyclase (Di Virgilio et al., 2001; Harris et al., 2002; Jutel et al., 2002; Pozo, 2003). However, the mechanism for cAMP-mediated immune suppression has not been elucidated. Here, we showed that the suppression works through the upregulation of the c-Fos protein, which, in turn, prevents the transcription of proinflammatory cytokines such as TNF-α. We also demonstrated that IKKβ-mediated phosphorylation and stabilization of c-Fos are also keys for this mechanism.

Because c-Fos forms a family with FosB, Fra-1, and Fra-2, one could argue whether other c-Fos family proteins could replace c-Fos. We noticed that these related genes were not highly induced in RAW264.7 cells (data not shown), which may explain why siRNA for *Fos* almost completely abrogated the effect of cAMP in RAW264.7 cells. However, *Fos* gene disruption did not completely prevent the cAMP effect in fetal liver macrophages. This discrepancy could be due to compensation by other Fos family proteins because other Fos family member proteins (FosB, Fra-1, and Fra-2) were upregulated in $Fos^{-/-}$ fetal-liver macrophages. Supporting this hypothesis, the overexpression of FosB or Fra-1 could suppress p65-induced *Tnf* promoter activation. These data suggest that not only c-Fos but also most other Fos family proteins are able to inhibit TNF-α mRNA induction when they are highly expressed.

c-Fos is a component of AP-1, which is usually thought to be involved in immune activation. For example, in T cells, AP-1 is known to enhance IL-2 transcription in cooperation with NF-AT (Jain et al., 1993). However, AP-1 is likely to be suppressive in innate immunity. A suppressive role of AP-1 in TLR system has been suggested in *Drosophila* (Kim et al., 2005). In mammals, the suppression of proinflammatory cytokine production by AP-1 has been suggested not only by cAMP-elevating agents but also by various stimuli, such as hepatocyte growth factor, macrophage-colony stimulating factor (M-CSF), and granulocyte macrophage-colony stimulating factor (GM-CSF), which all upregulate the ERK pathway (Puig-Kroger et al., 2001). Because the transcription of c-Fos is upregulated by the ERK pathway (Karin et al., 1997), c-Fos may be widely involved in the suppression of proinflammatory cytokine production by the ERK-activating agents.

What is the relationship between cAMP and IL-10? Both inhibit TNF-α and IL-12p40. We found that the effect of cAMP is independent of the IL-10 and STAT3 pathway. IL-10 did not upregulate the c-Fos protein (data not shown). Conversely, cAMP upregulated IL-10 production, suggesting that IL-10 partly contributes to the cAMP-mediated suppression in normal conditions. However, there are some differences between cAMP and IL-10; cAMP suppresses IFN-β mRNA induction, but not that of IL-6, whereas IL-10 suppresses IL-6 transcription, but not that of IFN-β. The molecular mechanism of proinflammatory cytokine suppression by the IL-10 and STAT3 pathway remained to be elucidated. Interestingly, it has been reported that COX2 inhibitor treatment of $Il10^{-/-}$mice resulted in the rapid development of more severe colitis compared with untreated $Il10^{-/-}$ mice (Berg et al., 2002), suggesting that endogenous prostaglandins are important inhibitors of the development of intestinal inflammation in $Il10^{-/-}$ mice. Therefore, the PGE2-cAMP system and the IL-10 system are independent safeguards for preventing excessive inflammation. These two pathways may also cooperatively suppress inflammation.

p50 often forms a heterodimer with p65 and regulates various NF-κB-mediated gene expression. However, it has been shown that p50 inhibits TNF-α production and that induction of p50 is a mechanism of LPS tolerance (Bohuslav et al., 1998). Induction of p50 may be a mechanism of the suppression by cAMP. However, p50 expression did not differ between LPS and LPS+cAMP. Thus, suppression of p65 activity on the *Tnf* promoter by c-Fos is not by induction of p50.

The results of our study were consistent with the previous report by Stein et al. (1993) showing that c-Fos protein directly interacts with the p65. We further demonstrated that this interaction reduced recruitment of p65 to the *Tnf* promoter region, which can account for the suppressive effect of cAMP. Binding of p65 to the −670 κB site of the *Tnf* promoter region was apparently reduced by the presence of c-Fos. This −670 κB site (GTGAATTCCC) is highly conserved between humans and rodents. The first half of this putative NF-κB binding site is different than the consensus NF-κB (p50:p65 heterodimer) recognition sequence (GGGRNTTTCC) and is probably preferentially recognized by p65:p65 homodimer (Chen et al., 1998). We confirmed the lack of p50 binding to this site by DNAP assay. A possible role of p65:p65 homodimer in LPS-induced *Tnf* promoter activation has been reported (Kuprash et al., 1995). From those observations, we propose that p65:p65 homodimer recognizes this critical GTGAATTCCC site, and the binding to this sequence is blocked by the binding of c-Fos or AP-1 complex to p65:p65 homodimer. Although c-Fos did not affect binding and transcriptional activity of p65:p50 heterodimer for the consensus κB sequence, we still do not know why binding of c-Fos to p65:p65 homodimer, but not p65:p50 heterodimer, reduced the binding affinity to DNA. However, we think that this model is quite attractive because it can explain how c-Fos selectively affects NF-κB-dependent promoter activation.

Our results also demonstrate that c-Fos protein accumulation is mediated by both LPS and cAMP signaling. Induction of c-Fos mRNA was mainly induced by a cAMP-wortmannin-sensitive kinase pathway, and stabilization and accumulation of c-Fos protein were mediated by the LPS-IKKβ pathway, which directly phosphorylated both the N- and C-terminal regions of c-Fos proteins. The c-Fos protein is very unstable, though it is stabilized by phosphorylation (Chen et al., 1993; Coronella-Wood et al., 2004; Murphy et al., 2002; Okazaki and Sagata, 1995; Sasaki et al., 2006; Tanos et al., 2005; Terasawa et al., 2003). These reports have shown that serine-threonine kinases, such as ERK, RSK, and Mos, stabilize c-Fos by phosphorylating S32, T325, T331, S362, and S374. We generated deletions and substitution mutants for these Ser-Thr residues. However, none of them affected phosphorylation by IKKβ in vitro and stabilization by IKKβ in HEK293T cells (data not shown). The current study identified a phosphorylation site, S308 of c-Fos by IKKβ. To our knowledge, this phosphorylation site has not been reported previously and differs from the consensus amino acid sequence

(DS[p]GXXS[p]) of IκBs and others phosphorylated by IKKβ (Scheidereit, 2006). Although c-Fos (S308D) was more stable than WT c-Fos before LPS stimulation or without coexpression of IKKβ, c-Fos (S308A) was still stabilized by IKKβ. Because IKKβ also phosphorylates the N-terminal region of c-Fos, other phosphorylation sites seem to be involved in full stabilization of c-Fos protein. Therefore, further study is necessary to completely understand the mechanisms of c-Fos stabilization by IKKβ or LPS.

In conclusion, we propose that c-Fos and its family proteins play important regulatory roles in innate immune systems. The induction of c-Fos proteins could be therapeutic for preventing acute and chronic inflammatory diseases, such as septic shock or autoimmune diseases.

## EXPERIMENTAL PROCEDURES

### Cells, Antibodies, and Reagents

Bone marrow-derived dendritic cells (BMDCs) were prepared from mouse bone marrow cells as described (Hanada et al., 2005). RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 10% fetal bovine serum, antibiotics (GIBCO), and nonessential amino acids (GIBCO). LPS (*Escherichia coli* serotype 055:B5) and 8-Bromoadenosine 3, 5′-cyclic monophosphate sodium salt (8-Br-cAMP) (B7880) were purchased from Sigma. Prostaglandin E2 (PGE2), cycloheximide, and Actinomycin D were purchased from Nacalai Tesque. Anti-c-Fos (sc-52), anti-p65 (sc-372), anti-p50 (sc-1192), anti-c-Jun (sc-1694), anti-IKKγ (sc-8330), anti-HA polyclonal (sc-805), anti-GST (sc-138), and anti-GFP (sc-8334) antibodies were purchased from Santa Cruz Biotechnology. Anti-Actin antibody (A2066) and anti-FLAG M2 antibody (F3165) were purchased from Sigma. Anti-HA (16B12) monoclonal antibody (MMS-101P) was purchased from Covance. Anti-IKKβ antibody (2678) was purchased from Cell Signaling Technology. LY294002, wortmannin, and IKK-2 inhibitor IV were obtained from Calbiochem. Calf intestinal alkaline phosphatase (CIAP) was obtained from TOYOBO.

### Plasmids

Murine *p65*, murine *Traf6*, and human *Fos* cDNAs were amplified by RT-PCR. The cDNAs were inserted into a pCMV vector (Takaesu et al., 2000), pCMV2-FLAG vector (Sigma), or pcDNA3.1 vector (Invitrogen) to generate pCMV/FLAG-p65, pCMV2/FLAG-TRAF6 and pCMV/FLAG-c-Fos, and pcDNA3.1/HA(x2)-c-Fos, respectively. The various lengths of c-Fos deletion fragments were amplified by PCR to generate pGEX5x-1/c-Fos deletion variants. The point-mutated c-Fos mutants were generated by primers as follows: S308A, 5′-CCTCTGCACgctGGCTCCCTGGGGATG-3′ and 5′-CATCCCCAGGGAGC CagcGTGCAGAGG-3′; S310A, 5′-CACAGTGGCgccCTGGGGATG-3′ and 5′-CATCCCCAGggcGCCACTGTG-3′, S308/S310A; 5′-CCTCTGCACgctGGC gccCTGGGGATG-3′ and 5′-CATCCCCAGggcGCCagcGTGCAGAGG-3′; S308D, 5′-CCTCTGCACgatGGCTCCCTGGGGATG-3′ and 5′-CATCCCCAGG GAGCCatcGTGCAGAGG-3′.

The FLAG-c-Fos WT, S308A or S308D fragment from pCMV/FLAG-c-Fos, and the IRES-EGFP fragment from the pGCDΔNsamI/E vector (Nishinakamura et al., 2007) were inserted into the pcDNA3.1 vector (Invitrogen) to generate pcDNA3.1/FLAG-c-Fos (WT, S308A or S308D)-IRES-EGFP. The GST-IκBα (1–72) was kindly provided by Dr. T. Ishitani (Kyushu University). The murine *Tnf* promoter −2.0 kb region was amplified by RT-PCR with the following primers: forward, 5′-GCGCGCTAGCGCTCCCCCAGGGCCATT-3′ (−2.0 kb), 5′-GCGCGCAGCCAGAGTGAAAGGAGAAGGCT-3′ (−0.7 kb), and 5′-GCGC GCTAGCTCATTCCCTCTGGGGCTGCC-3′ (−0.65 kb); and reverse, 5′-GCGCAAGCTTGGTGTCTTTTCTGGAGGGAG-3′. The amplified DNA fragments were inserted into the Pickagene basic vector (TOYO Ink) to generate the *Tnf* promoter-luciferase vector.

### Mice and Primary Cells

*Il10*$^{-/-}$ mice, *Stat3*$^{fl/fl}$, and LysM-Cre/*Fos*$^{fl/fl}$ mice were obtained from Dr. K. Takeda (Osaka University). The *Fos*$^{-/-}$ mice have been described previously (Wang et al., 1992). Dendritic cells from bone marrow were generated as described (Hanada et al., 2005). The macrophages derived from fetal liver obtained by a modified method have also been described previously (Mason et al., 2004). All experiments with these mice were approved by and performed according to the guidelines of the Animal Ethics Committee of Kyushu University, Fukuoka, Japan.

### ELISA and RT-PCR

ELISA was performed with an ELISA kit (eBioscience). The total RNA of cells was prepared with the RNAiso reagent (TAKARA), and RT-PCR was carried out with the one-step RT-PCR kit (Applied Biosystems). Real-time PCR was carried out with SYBR-Green (Applied Biosystems). The following oligonucleotides were used: G3PDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCA CCACCCTGTTGCTGTA-3′; TNF-α, 5′-TGAACTTCGGGGTGATCGGTC-3′ and 5′-AGCCTTGTCCCTTGAAGAGGAC-3′; IL-6, 5′-GACTTCACAGAGGATAC CAC-3′ and 5′-TCTCTCTGAAGGACTCTGGC-3′; IL-12p40, 5′-GTAGAGGT GGACTGGACTCC-3′ and 5′-GCAGACAGAGACGCCATTCC-3′; IL-10, 5′-GG ACAACATACTGCTAACCG-3′ and 5′-TTCATGGCCTTGTAGACACC-3′; IFN-β, 5′-TCCAAGAAAGGACGAACATTCG-3′ and 5′-TGAGGACATCTCCCACG TCAA-3′; c-Fos, 5′-CTGTCCGTCTCTAGTGCCAACTT-3′ and 5′-ATCTGTC TCCGCTTGGAGCGTAT-3′; and HA-c-Fos, 5′-CCTACGATGTGCCCGATT AT-3′ and 5′-TGGCTGCAGCCATCTTATTC-3′.

### Microarray Analysis

Microarray analysis was performed as described (Hanada et al., 2005). In brief, BMDCs were unstimulated or stimulated with LPS (10 ng/ml) alone or with LPS+cAMP (100 μM). The total RNA of BMDCs was isolated with Trizol (Invitrogen), and biotinylated cRNAs were synthesized with a one-cycle target-labeling kit (Affymetrix). Fragmented cRNAs were hybridized to the Mouse 430 2.0 GeneChip (Affymetrix) and analyzed by GeneSpring.

### Intracellular Staining

RAW264.7 cells were transfected with pcDNA3.1/FLAG-c-Fos-IRES-EGFP or an empty EGFP vector. At 24 hr later, cells were stimulated with 10 ng/ml of LPS and Brefeldin A for 6 hr. Cells were collected and fixed with an intracellular staining kit (eBioscience) and stained with the anti-TNF-α-PE antibody and analyzed by FACScalibur as previously described (Nishinakamura et al., 2007).

### Reporter Gene Analysis

RAW264.7 cells were seeded onto 6-well plates 1 day before transfection as described (Nishinakamura et al., 2007). The *Tnf* promoter luciferase vector was cotransfected with cDNAs together with the β-galactosidase control plasmid with FuGENE HD (Roche). The luciferase activities were measured with the luciferase assay system (Promega) and normalized by internal control β-gal activity.

### RNAi Analysis

siRNA (50 nM) was transfected by Lipofectamine RNAi MAX (Invitrogen) into RAW264.7 cells. The following siRNAs (sense strands) were used for RNAi analysis for c-Fos and IKKβ: c-Fos, GCAGATCTGTCCGTCTCTA; IKKβ, GGA CATCGTTGTTAGTGAA.

### Adoptive Transfer of DCs

DCs from spleen cells were untreated or stimulated with OVA peptide (10 μg/ml) and LPS (10 ng/ml). 1 × 10$^6$ cells were injected intravenously into OT-II mice. After 7 days, lymph node cells were isolated, and 1 × 10$^5$ cells were seeded into 96-well plates. Cells were stimulated with PMA and ionomycin for 6 hr, and the concentrations of IFN-γ were measured by ELISA.

### ChIP Assay

The chromatin immunoprecipitation (ChIP) assay was performed as previously described (Kinjyo et al., 2006) with a ChIP assay kit (Upstate Biotechnology). Briefly, RAW264.7 cells were fixed with 1% formaldehyde at 37°C for 5 min and then suspended in an SDS lysis buffer. After sonication with 30 s pulses 10 times with a sonicator (Bioruptor; Cosmo Bio.), samples were incubated with 5 μg antibodies or control IgG overnight at 4°C. After the addition of salmon sperm DNA and ProteinA-Agarose Slurry, the immunoprecipitates were sequentially washed once with a low-salt buffer, a high-salt buffer, and

an LiCl buffer and twice with a TE buffer. The DNA-protein complex was eluted by heating at 65°C for 6 hr. Proteins were then digested by proteinase K, and RNA was removed by the addition of 10 μg of RNase A. DNA was recovered by phenol/chloroform extraction and ethanol precipitation and then subjected to PCR analysis. The following oligonucleotides were used to amplify the mouse *Tnf* and mouse *Il6* promoter regions: *Tnf* (−700 to −490 bp), 5′-GCCACTTCCTCCAAGAACT-3′ and 5′-TTGGAAAGTTGGGGACACC-3′; *Tnf* (−800 to −600 bp), 5′-CTGTGGGACCTAAATGTCTG-3′ and 5′-GGGTAATGGGATGAGTATGG-3′; and *Il6* (−307 to −72 bp), 5′-TGCTCAAGTGCTGAGTCACT-3′ and 5′-AGACTCATGGGAAAATCCCA-3′.

### DNA Affinity Purification Assay

The DNA affinity precipitation assay (DNAP) was performed as described (Suzuki et al., 1993). Briefly, 50 μg nuclear extracts were incubated with biotin-labeled oligonucleotides (0.5 μg) and poly dI-dC (5 μg) for 30 min in a DNAP buffer. Then, Streptavidin-Dynabeads M-280 were mixed and rotated for 1 hr at 4°C. The precipitated proteins were analyzed by SDS-PAGE. The following biotin-labeled oligonucleotides were used: NF-κB consensus, 5′-GGATCAGGGACTTTCCGCTGGGGACTTTCCG-3′; *Tnf* promoter (−670 to −643 bp), 5′-GTACGTGAATTCCCAGGGCTGAGTTCATTC-3′; STAT6-binding site, 5′-GATCGCTTTTTTCCTCTAAACTGC-3′.

### In Vitro Kinase Assay

The IKK in vitro kinase assay was performed according to the procedure described previously (Takaesu et al., 2001). IKKβ was expressed in HEK293T cells transfected with FLAG-IKKβ or FLAG-IKKβ KN and was immunoprecipitated by anti-FLAG (M2) antibody. IKK complex was also prepared from RAW264.7 cells that were stimulated with LPS (100 ng/ml) for 1 hr by immunoprecipitation with anti-IKKγ antibody. GST proteins were purified by glutathione Sepharose. Immunoprecipitates and 1 μg of GST proteins were incubated with 5 μCi of [γ-$^{32}$P]-ATP at 30°C for 5 min in kinase buffer (10 mM HEPES-KOH [pH 7.4], 5 mM MgCl2, and 1 mM DTT). The phosphorylated proteins were detected by SDS-PAGE followed by CBB staining and autoradiography.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and eight figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00103-4.

## ACKNOWLEDGMENTS

We thank K. Takeda (Osaka University) for providing *Il10*$^{-/-}$ and *STAT3*-flox mice; S. Okada (Kumamoto University) for *Fos*$^{-/-}$ mice; Y. Nakabeppu (Kyushu University) for c-Fos, FosB, and Fra1 cDNAs; and T. Ishitani (Kyushu University) for GST-IκBα plasmid. We also thank T. Yoshioka, M. Ohtsu, K. Fukidome, and M. Asakawa for technical assistance and N. Soma and Y. Nishi for manuscript preparation. This study was supported by special Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); Astellas Foundation for Research on Metabolic Disorders; the Japan Intractable Disease Research Foundation; the Mochida Memorial Foundation; the Naito Memorial Foundation; and the Princess Takamatsu Cancer Research Fund.

Received: August 14, 2008
Revised: December 4, 2008
Accepted: December 30, 2008
Published online: March 12, 2009

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