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## NF- $\kappa$ B1 Inhibits TLR-Induced IFN- $\beta$ Production in Macrophages Through TPL-2-dependent ERK Activation<sup>1</sup>

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### Abstract

Although NF- $\kappa$ B1 p50/p105 has critical roles in immunity, the mechanism by which NF- $\kappa$ B1 regulates inflammatory responses is unclear. Here, we analyzed the gene expression profile of lipopolysaccharide (LPS)-stimulated *Nfkb1*<sup>-/-</sup> macrophages that lack both p50 and p105. Deficiency of p50/p105 selectively increased the expression of interferon (IFN)-responsive genes, which correlated with increased IFN- $\beta$  expression and STAT1 phosphorylation. Interferon antibody blocking experiments indicated that increased STAT1 phosphorylation and expression of interferon-responsive genes observed in the absence of p50/p105 depended upon autocrine IFN- $\beta$  production. Markedly higher serum levels of IFN- $\beta$  were observed in *Nfkb1*<sup>-/-</sup> mice than in WT mice following LPS injection demonstrating that *Nfkb1* inhibits IFN- $\beta$  production under physiological conditions. TPL-2, a mitogen-activated protein kinase kinase kinase stabilized by association with the C-terminal ankyrin repeat domain of p105, negatively regulates LPS-induced interferon- $\beta$  production by macrophages via activation of ERK MAP kinase. Retroviral expression of TPL-2 in *Nfkb1*<sup>-/-</sup> macrophages, which are deficient in endogenous TPL-2, reduced LPS-induced interferon- $\beta$  secretion. Expression of the C-terminal ankyrin repeat domain of p105 in *Nfkb1*<sup>-/-</sup> macrophages, which rescued LPS activation of ERK also inhibited IFN- $\beta$  expression. These data indicate that p50/p105 negatively regulates LPS induced interferon-signaling in macrophages by stabilizing TPL-2, thereby facilitating activation of ERK.

### Introduction

The NF- $\kappa$ B family of transcription factors, which share an N-terminal Rel-homology domain, includes five subunits, NF- $\kappa$ B1 p50, p65 (RelA), c-Rel, RelB, and NF- $\kappa$ B2 p52 (1). These proteins form homo and hetero-dimers that are retained in the cytoplasm of unstimulated cells by members of the I $\kappa$ B family of ankyrin repeat containing proteins (I $\kappa$ B-

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$\alpha$ ,  $\beta$ , and  $\epsilon$ ) (2). Inflammatory stimuli activate the I $\kappa$ B kinase complex (IKK)<sup>4</sup>, which phosphorylates two serines found within the N-termini of the I $\kappa$ Bs, directing proteosomal degradation (3). I $\kappa$ B degradation allows nuclear translocation of liberated NF- $\kappa$ B dimers, which then modulate transcription by binding to  $\kappa$ B sites found within the promoter and enhancer regions of many genes involved in immune and inflammatory responses (1,4). p65, c-Rel, and RelB, which each have a C-terminal trans-activation domain, are synthesized in their mature form. In contrast, p50 and p52 are produced from the N-termini of larger precursor proteins NF- $\kappa$ B1 p105 and NF- $\kappa$ B2 p100, respectively, by proteolytic removal of their C-termini by the proteasome, and lack transactivation domains (2).

NF- $\kappa$ B1 p50 induces transcription in heterodimeric complexes with transactivating Rel subunits. However, p50 homodimers can repress transcription by interfering with the binding of other active NF- $\kappa$ B dimers and recruiting histone deacetylases to the promoter regions of  $\kappa$ B-target genes (5,6). It has been suggested that p50 homodimers are responsible for inhibiting tumor necrosis factor (TNF) expression in LPS tolerized macrophages (7–9), and maintaining an anti-inflammatory phenotype in M2 polarized tumor-associated macrophages (9,10). p105 also has NF- $\kappa$ B inhibitory activity, due to its C-terminal ankyrin repeats. These are homologous to those found in the classical I $\kappa$ B proteins, and retain NF- $\kappa$ B dimers in the cytoplasm of unstimulated cells. IKK phosphorylation of two serines in the C-terminal region of p105 after agonist stimulation leads to proteasomal-dependent complete degradation of p105 (11–13), releasing associated p50, p65, and c-Rel to translocate into the nucleus (14). The C-terminal portion of p105 additionally binds to TPL-2 (also known as MAP3K8 and Cot) (11,15), a mitogen-activated protein kinase kinase that is essential for ERK activation following LPS stimulation of macrophages (16). Binding of p105 prevents TPL-2 from phosphorylating its downstream targets, MEK-1/2, and is also required to maintain TPL-2 protein stability. Consequently, LPS-induced activation of ERK-1/2 is blocked in bone marrow-derived macrophages (BMDM) that lack p50/p105, due to substantially decreased amounts of TPL-2 protein (17).

The multiple biochemical functions of NF- $\kappa$ B1 p50/p105 suggest that its role in immune responses is complex. This is consistent with observations that mice lacking p50/p105 exhibit both multi-focal immune defects and increased sensitivity to inflammation (18,19). To further define the function of p50/p105 during Toll-like receptor (TLR)-induced inflammatory responses, we profiled gene expression following LPS stimulation of *Nfkb1*<sup>-/-</sup> macrophages. This analysis revealed that macrophages lacking p50/p105 demonstrated augmented induction of a type I interferon response. We show that the ability of NF- $\kappa$ B1 p50/p105 to inhibit this response depends on its ability to stabilize TPL-2, and facilitate TLR-induced ERK activation. Thus, p50/p105-dependent ERK activation plays an important role in regulating the nature of TLR-induced inflammatory responses.

## Materials and Methods

### Experimental Animals

*Il10*<sup>-/-</sup>, *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup> and *Il10*<sup>-/-</sup>*Rag2*<sup>-/-</sup> mice were maintained on the 129S6/SvEvTac background, as previously described (20,21). *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup>*Rag*<sup>-/-</sup> mice were generated by crossing *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup> and *Il10*<sup>-/-</sup>*Rag2*<sup>-/-</sup> mice. *Map3k8*<sup>-/-</sup> mice (kindly provided by Professor Philip Tschlis, Tufts University, Boston and Thomas Jefferson University, Philadelphia) were on the C57BL/6 background. In experiments comparing *Map3k8*<sup>-/-</sup> mice with wild type (WT) and *Nfkb1*<sup>-/-</sup> mice, all strains were on the C57BL/6 background.

<sup>4</sup>Abbreviations: IKK, I $\kappa$ B kinase complex; BMDM, bone marrow-derived macrophages.

### **In vitro stimulation of bone marrow-derived macrophages**

BMDM were grown as previously described (20) and stimulated with LPS from *E. coli* 0127:B8 (Sigma, St Louis, MO) at 1 ng/ml (unless stated otherwise) or mouse IFN- $\beta$  (PBL Biomedical Laboratories, New Brunswick, New Jersey) at 10 U/ml. rIL-10 (BD Biosciences, San Diego, CA) was used at 0.3 ng/ml. IFN- $\beta$  depletion assays were performed by including anti-IFN- $\beta$  antibody (75-D3, Yamasa Soya, corp., Tokyo, Japan) or control IgG at a concentration of 6.7 $\mu$ g/ml. PD184352 (kindly provided by Professor Sir Philip Cohen, University of Dundee, UK) was used at a concentration of 2 $\mu$ M.

### **Preparation of RNA**

RNA was isolated in Trizol (Invitrogen, Carlsbad, CA) or using RNeasy Mini Kit (Quiagen, Valencia, CA) following the manufacturer's instructions.

### **Gene Expression Profiling**

Probes were prepared from isolated RNA, and hybridized to Affymetrix 430A 2.0 arrays. Two independent samples were analyzed for each condition. Expression data were read from CEL files and background correction, normalization, and summarization of the probe-level data into probe set expression values were accomplished using Robust Multi-Array Analysis (RMA). Differential expression between samples was analyzed using the LIMMA package of the afflylmGUI within the Bioconductor R environment (22). The microarray data discussed in this publication are accessible through National Center for Biotechnology Information's Gene Expression Omnibus, accession number GSE19941 (<http://www.ncbi.nlm.nih.gov/geo>).

### **RT-PCR**

RT-PCR analysis was performed using probes from Applied Biosystems (Foster City, CA), as per the manufacturer's instructions. Expression for each sample was analyzed in duplicate and normalized between samples using the  $\Delta\Delta$  cycle threshold method. Fold-change is reported as relative to uninduced levels in control BMDM. Each data point represents the average  $\pm$  SEM for 2 or 3 independent macrophage pools. Experiments are representative of 2 or 3 independent experiments with consistent results.

### **ELISA**

IFN- $\beta$  was analyzed in culture supernatants and serum using a commercially available IFN- $\beta$  specific ELISA kit (PBL Biomedical Laboratories), according to the manufacturer's instructions.

### **Immunoblotting**

ERK (p44/42 MAP Kinase), phospho ERK (p44/42 MAP Kinase) (Thr202/Tyr204), Phospho-STAT1 (Y701) antibodies were purchased from Cell Signaling (Beverly, MA). STAT1 p84/p91 (E-23), TFIID (SI-1), c-Fos (sc-52), and TPL-2 (sc-726) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### **In vivo LPS challenge**

Adult male mice (10–12 weeks old) were injected intraperitoneally with LPS (200  $\mu$ g) and sacrificed 4h later. Serum was collected and IFN- $\beta$  was measured by ELISA.

### **Retrovirally-mediated gene expression**

For expression of Tpl-2 and c-Fos, BMDM were infected with ecotropic retroviruses expressing the gene of interest and GFP, or GFP alone, as described (23). Following

infection, GFP-positive BMDM were isolated by fluorescence-activated cell sorting. Cells were cultured at  $2 \times 10^5$  cells/well in 200  $\mu$ l of medium in a 96-well plate and stimulated with 10 ng/ml LPS for 24h. The culture medium was used for IFN- $\beta$  specific ELISA, and cell pellets were lysed in 50 $\mu$ l lysis buffer (26) for measurement of total protein by Bradford assay (Biorad). For expression of p105 $\Delta$ N, BMDM were infected with retroviruses expressing p105 $\Delta$ N (murine p105 amino acids 434–971) and the gene for puromycin resistance, or the gene for puromycin resistance alone. Puromycin resistant cells were isolated by selection for 72 hours in media containing puromycin. After selection, cells were replated at  $5 \times 10^5$  cells/well in 1 ml of medium in a 24-well plate for total protein extraction, and at  $5 \times 10^4$  cells/well in 200  $\mu$ l of media for analysis of cytokine secretion. Cells were stimulated the following day with LPS. Culture supernatants were assayed for IFN- $\beta$  by ELISA.

### p50 DNA Binding ELISA

p50 DNA binding activity in nuclear extracts derived from LPS stimulated WT and *Map3k8*<sup>-/-</sup> BMDM was measured with the p50 DNA binding ELISA kit from Active Motif (Carlsbad, CA), as per the manufacturer's instructions. Data are normalized to total protein in the nuclear extracts determined by Bradford assay (Biorad).

### Statistical Analysis

All data analysis was performed using GraphPad Prism software (GraphPad Software, inc., San Diego, CA). Data generated by RT-PCR or ELISA was compared using t-tests for parametric data. \* indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ , and \*\*\* indicates  $P < 0.001$ . Error bars represent SEM.

## Results

### Profiling LPS-induced gene expression in p50/p105-deficient macrophages

To characterize the role of p50/p105 in regulating inflammatory gene expression, we profiled LPS-induced gene expression in BMDM lacking p50/p105 and IL-10 (*Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup>). Experiments were performed with BMDM additionally lacking IL-10 because LPS-induced expression of endogenous IL-10 has a strong inhibitory effect on the expression of many inflammatory genes (24), and there are marked differences in levels of IL-10 secretion following TLR stimulation of WT and p50/p105-deficient macrophages (25–27). Low levels of exogenous IL-10 were added to cultures to amplify differences in expression between *Il10*<sup>-/-</sup> and *Nfkb1*<sup>-/-</sup> *Il10*<sup>-/-</sup> BMDM (20).

Compared to expression in unstimulated cells, 216 out of 22,626 probe sets evaluated were induced at least 4-fold by LPS in either *Il10*<sup>-/-</sup> or *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup> BMDM (Table S1). Expression levels were significantly higher for 121 of these 216 LPS-induced probe sets in *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup> compared with *Il10*<sup>-/-</sup> BMDM, while 16 were expressed at significantly lower levels. The 20 genes with the largest fold-change between LPS stimulated *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup> and *Il10*<sup>-/-</sup> BMDM are shown in Table I. A significant fraction of these were type I interferon-responsive genes, including *Mx1* and *Cxcl9* (28,29). This suggested that p50/p105 might preferentially inhibit expression of IFN-responsive genes following LPS stimulation.

### p50/p105 inhibits TLR-induced IFN-responsive gene expression

To determine whether p50/p105 deficiency selectively increased IFN responsive gene expression in the absence of exogenous IL-10, we next analyzed a select set of LPS-inducible genes by RT-PCR. These genes included three that have previously been reported as IFN-responsive (*Mx1*, *Cxcl9*, and *Nos2*) (28–30), and three that are not IFN-responsive

(*Cxcl1*, *Cxcl2*, and *Il1b*). These experiments were performed in BMDM that also lack the gene for RAG2, as we found that lymphocyte-sufficient mice lacking both p50 and IL-10 spontaneously develop inflammatory disease as they age (BHH, unpublished data). Consistent with the gene profiling data, mRNAs encoding IFN-responsive genes were expressed at significantly higher levels in *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM than in *Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM after LPS stimulation (Fig. 1A), while the mRNAs encoding genes that are not IFN-responsive were expressed at similar or lower levels (Fig. 1B). Expression of *Mx1*, *Cxcl9*, and *Nos2* mRNAs were induced to comparable levels in *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* and *Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM following stimulation with exogenous IFN- $\beta$ , while *Cxcl1*, *Cxcl2*, and *Il1b* were not induced in cells of either genotype (Fig. 1C). These results confirmed that p50/p105 suppressed the ability of LPS to induce expression of IFN-responsive genes, and also demonstrated that p50/p105 did not regulate the sensitivity of macrophages to IFN- $\beta$  stimulation. Furthermore, these data indicated that p50/p105 had inhibitory functions that were independent of the regulation of, or response to, IL-10.

We next determined whether the increase in IFN-responsive gene expression observed following LPS stimulation of BMDM lacking p50/p105 could result from increased production of IFN- $\beta$  itself. Consistent with this hypothesis, we found that LPS induced significantly higher expression of *Ifnb1* mRNA, as well as higher levels of secreted IFN- $\beta$  protein, in *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM than in *Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM (Fig. 1D).

### Increased IFN-signaling observed in the absence of p50/p105 depends on expression of IFN- $\beta$

The biological effects of IFN- $\beta$  are mediated largely through activation of STAT1. LPS-induced phosphorylation of STAT1 was significantly augmented in the absence of p50/p105 (Fig. 2A), and this was markedly inhibited by treatment of *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM with an IFN- $\beta$  blocking antibody (31) (Fig. 2B). Antibody treatment also impaired LPS-induced expression of IFN-responsive genes *Cxcl9* and *Nos2*, while having little effect on expression of IFN- $\beta$  itself (Fig. 2C). These results suggest that elevated expression of IFN-dependent genes in macrophages lacking p50/p105 depended on autocrine IFN- $\beta$  stimulation and augmented activation of STAT1. These data are consistent with a recent report showing increased LPS-induced IFN- $\beta$  and IFN-dependent signaling in LPS-stimulated peritoneal exudative cells isolated from *Nfkb1<sup>-/-</sup>* mice (9).

### LPS induces increased serum levels of IFN- $\beta$ in *Nfkb1<sup>-/-</sup>* mice

To determine whether p50/p105 suppresses the induction of IFN- $\beta$  following inflammatory challenge in vivo, WT and *Nfkb1<sup>-/-</sup>* mice on the C57BL/6 background received 200  $\mu$ g of LPS i.p.. Serum was harvested 4 hours later and levels of IFN- $\beta$  determined by ELISA. LPS induced detectable levels of IFN- $\beta$  in the serum of *Nfkb1<sup>-/-</sup>* mice but not in WT mice (Fig. 3). This demonstrates that p50/p105 suppresses the systemic induction of IFN- $\beta$  following LPS challenge.

### Inhibition of TLR-induced IFN-signaling by p50/p105 depends on TPL-2

LPS-induced ERK activation is blocked in *Nfkb1<sup>-/-</sup>* BMDM due to TPL-2 deficiency (11,17,32), and we have recently shown that the TPL-2/ERK signaling pathway negatively regulates LPS induction of IFN- $\beta$  in macrophages (26). This raised the possibility that defective TPL-2/ERK signaling was responsible for increased IFN- $\beta$  signaling observed in p50/p105-deficient BMDM. Indeed, in addition to the expected increase in IFN- $\beta$  expression (Fig. S1A), we detected increased STAT1 phosphorylation (Fig. 4A) and increased IFN-dependent gene expression (Fig. 4B) in TPL-2-deficient (*Map3k8<sup>-/-</sup>*) BMDM compared to WT BMDM following LPS stimulation. Therefore, the phenotype of TPL-2-deficient *Map3k8<sup>-/-</sup>* macrophages was very similar to that of p50/p105-deficient macrophages.

To investigate whether the effect of TPL-2 deficiency on type I IFN signaling was due to impaired activation of ERK, WT BMDM were pre-treated with the MEK inhibitor PD184352 prior to stimulation with LPS. Blockade of ERK activation with PD184352 (Fig. 5A) increased STAT1 phosphorylation (Fig. 5B), and IFN-responsive gene expression (Fig. 5C), as well as increasing expression of IFN- $\beta$ , as previously described (26) (Fig. S1B). Therefore, the effects of TPL-2 deficiency on IFN signaling were phenocopied by pharmacological blockade of ERK activation, consistent with the critical role of TPL-2 in regulating ERK activation in TLR-stimulated macrophages (see Fig. 4A).

To test the hypothesis that decreased levels of TPL-2 in p50/p105-deficient BMDM were responsible for enhanced expression of *Ifnb1* following LPS stimulation, p50/p105-deficient BMDM were infected with a retrovirus encoding both TPL-2 and GFP, to increase TPL-2 protein levels (23), or control vector encoding GFP alone. After sorting for GFP-positive cells, BMDM were stimulated with LPS, and the production of IFN- $\beta$  evaluated by ELISA. Infection with the virus expressing TPL-2 markedly inhibited induction of IFN- $\beta$  compared to infection with control virus (Fig. 6A). These results indicate that impaired activation of the TPL-2/ERK signaling pathway in p50/p105-deficient BMDM is responsible for increased IFN-signaling observed in these cells.

It has been previously shown that the C-terminal ankyrin repeat region of p105 is necessary to stabilize TPL-2 and facilitate ERK activation in LPS stimulated macrophages (11,17,33). Therefore, our results imply that the C-terminal region of p105 has essential functions necessary to inhibit *Ifnb1* expression that are independent of any putative inhibitory function of p50 homodimers. Consistent with this hypothesis, TPL-2-deficient *Map3k8*<sup>-/-</sup> BMDM exhibit normal levels of active p50 after LPS stimulation, as detected by NF- $\kappa$ B ELISA (Fig. 6B). Furthermore, the observation that *Map3k8*<sup>-/-</sup> and *Nfkb1*<sup>-/-</sup> macrophages produce similar levels of IFN- $\beta$  after LPS stimulation (Fig. 6C) suggests that the role of p50 homodimers may be relatively minor.

To directly investigate whether the C-terminal ankyrin repeat region of p105 is responsible for inhibition of IFN- $\beta$  expression, we infected BMDM derived from *Nfkb1*<sup>-/-</sup>*I110*<sup>-/-</sup>*Rag*<sup>-/-</sup> mice with either a control retrovirus (vector) or one expressing the C-terminal ankyrin repeat domain of p105 (p105 $\Delta$ N) tagged with hemagglutinin. Infected BMDM were selected with puromycin, and following replating at equal density, were stimulated with LPS. Immunoblotting of BMDM lysates confirmed successful expression of p105 $\Delta$ N (Fig. 7A), which rescued activation of ERK following LPS stimulation, as expected (Fig 7A). Further, in response to LPS, p105 $\Delta$ N markedly inhibited secretion of IFN- $\beta$  compared to cells infected with control vector (Fig. 7B). These results show that the C-terminal ankyrin repeat region of p105 inhibits induction of IFN- $\beta$  secretion following LPS challenge.

c-Fos is a member of the AP-1 transcription factor family that is induced in response to TLR ligation (34). Induction of c-Fos depends on ERK activation, and it has been demonstrated that c-Fos can inhibit expression of IFN- $\beta$ (26). c-Fos DNA binding activity is decreased following TLR stimulation of *Map3k8*<sup>-/-</sup> BMDM compared to WT cells (26), and defective induction of c-Fos protein expression has been observed following stimulation of p50/p105-deficient *Nfkb1*<sup>-/-</sup> BMDMs with *H. hepaticus* (32). Consistent with these previous reports, we observed a defect in the expression of c-Fos protein following LPS stimulation of *Nfkb1*<sup>-/-</sup> and *Map3k8*<sup>-/-</sup> BMDM (Fig. 8A). Furthermore, retroviral-mediated expression of c-Fos significantly inhibited IFN- $\beta$  LPS-induced IFN- $\beta$  secretion in *Nfkb1*<sup>-/-</sup> BMDM (Fig 8B). These results indicate that reduced induction of c-Fos following LPS stimulation may be responsible for augmented production of IFN- $\beta$  by p50/p105-deficient macrophages.

## Discussion

The results reported here demonstrate that p50/p105 suppresses the ability of LPS to induce IFN- $\beta$  expression both in macrophages in vitro, and in vivo following intraperitoneal injection. Increased IFN-responsive gene expression observed in the absence of p50/p105 is, at least in part, dependent on expression of IFN- $\beta$ , as IFN- $\beta$  depletion interferes with expression of IFN-responsive genes in p50/p105-deficient BMDM. These results are consistent with a recently published study demonstrating increased expression of IFN- $\beta$ , Cxcl9, and Nos2, as well as increased STAT1 activation in LPS-treated peritoneal exudative cells harvested from mice lacking p50/p105 (9). We have further shown that macrophages lacking TPL-2 also exhibit increased STAT1 activation and IFN-dependent gene expression following LPS stimulation, and that this is phenocopied by inhibition of ERK. These data are consistent with our earlier demonstration that the TPL-2/ERK signaling pathway negatively regulates TLR-induced IFN- $\beta$  production by macrophages (26). Retroviral expression of TPL-2 in *Nfkb1*<sup>-/-</sup> BMDM, which are deficient in endogenous TPL-2, inhibits LPS-induced induction of IFN- $\beta$ , indicating that impaired TPL-2-dependent ERK activation is responsible for increased IFN-expression and IFN-signaling observed in BMDM lacking p50/p105. Expression of the C-terminal ankyrin repeat domain of p105 in *Nfkb1*<sup>-/-</sup> macrophages, which reconstitutes LPS-induced ERK activation, also suppresses IFN- $\beta$  secretion, consistent with the role of the C-terminal domain in stabilizing the steady-state expression of TPL-2. Finally, expression of c-Fos in *Nfkb1*<sup>-/-</sup> BMDM inhibits IFN- $\beta$  secretion, suggesting that the reduction in ERK-dependent c-Fos expression observed in *Nfkb1*<sup>-/-</sup> BMDM, may be an important factor leading to elevated production of IFN- $\beta$ .

IL-10 expression is reduced in macrophages lacking p50/p105 compared to WT cells after TLR stimulation. Because IL-10 is a potent inhibitor of many TLR-inducible genes including *IFN- $\beta$*  and *CXCL9* (35), it is possible that increased expression of IFN-responsive genes observed following LPS stimulation of *Nfkb1*<sup>-/-</sup> BMDM could in part result from decreased production of IL-10. However, as our initial experiments were performed using macrophages that also lack the ability to secrete IL-10, differences in gene expression observed in this study could not be solely due to altered endogenous production of IL-10. p50/p105 therefore has inhibitory functions that are independent of IL-10. Consistent with this conclusion, we previously demonstrated that TPL-2/ERK signaling negatively regulates IFN- $\beta$  production in the absence of IL-10 (26).

It has been reported that macrophages lacking IKK- $\beta$  exhibited augmented activation of STAT1 following stimulation with heat-killed group B streptococcus (36). Since IKK- $\beta$  regulates activation of the TPL-2/ERK signaling pathway via phosphorylation of p105 (11,13), it might be expected that increased STAT1 activation in IKK- $\beta$ -deficient macrophages resulted from augmented IFN- $\beta$  production, as in *Nfkb1*<sup>-/-</sup> macrophages. Surprisingly, however, IFN- $\beta$  production was reported to be reduced in the absence of IKK- $\beta$  (36). These data suggest that IKK- $\beta$  likely has roles in both the induction, and p105-dependent inhibition, of IFN- $\beta$  expression following LPS stimulation. The observation that LPS-induced IFN- $\beta$  expression is lower in macrophages lacking IKK- $\beta$ , suggests that any decrease in activation of the p105-dependent inhibitory pathway does not overcome the reduction in the primary IKK/NF- $\kappa$ B-dependent induction of IFN- $\beta$  transcription. The mechanism for the negative regulation of STAT1 activation by IKK- $\beta$ , which is apparently independent of effects on IFN- $\beta$  production, to our knowledge, remains unknown.

Macrophages can exhibit divergent functional programs depending on the nature of the activating stimuli (37). Classic or M1 activation is induced by microbial products and/or IFN- $\gamma$ , which induce a pro-inflammatory response (increased IL-12, NOS2, MHC class II, and IFN- $\beta$  expression) required to kill intracellular pathogens. In contrast, M2 alternative

activation by IL-4 and IL-13 following parasite infection, is characterized by decreased expression of NOS2 and MHC class II, and increased expression of anti-inflammatory cytokines such as IL-10. Based on analyses of *Nfkb1*<sup>-/-</sup> macrophages, Porta et al. have proposed that p50 inhibits M1 polarization of LPS-stimulated macrophages (9). As over-expression of p50 in RAW 267.4 macrophages suppressed LPS-induced IFN- $\beta$  promoter activity, it was suggested that p50 homodimers might directly inhibit IFN- $\beta$  transcription (9). Our study indicates that the C-terminal ankyrin repeat domain of p105, which is known to interact with and stabilize TPL-2 (17), inhibits IFN- $\beta$  expression by facilitating LPS-induced ERK activation. These results are consistent with the hypothesis that defective TPL-2/ERK signaling is the predominant factor leading to elevated expression of IFN- $\beta$  in p50/p105-deficient macrophages, rather than the absence of p50. Our experiments further suggest that LPS-induced expression of c-Fos, which depends on the p105/TPL-2/ERK pathway, inhibits IFN- $\beta$  expression, providing a mechanism for the increase in IFN- $\beta$  expression observed in *Nfkb1*<sup>-/-</sup> macrophages.

In conclusion, this study shows that a major function for NF- $\kappa$ B1 p50/p105 in regulating gene expression in TLR-stimulated macrophages is to facilitate activation of the TPL-2/ERK signaling pathway by maintaining steady-state TPL-2 protein levels. These data raise the possibility that in vivo phenotypes of *Nfkb1*<sup>-/-</sup> mice that have been attributed to altered NF- $\kappa$ B function might actually be due, at least in part, to impaired activation of ERK MAP kinase in innate immune responses. This is supported by the observation that mice lacking only p105 are susceptible to colitis (38), similar to the colitis susceptible phenotype previously observed in mice lacking both p50 and p105 (18,20,27). Further studies will be necessary to define the relative importance of impaired activation of the TPL-2/ERK pathway versus altered NF- $\kappa$ B activation for the phenotype of *Nfkb1*<sup>-/-</sup> mice in models of infectious disease and autoimmunity.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

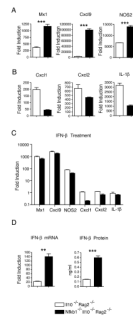
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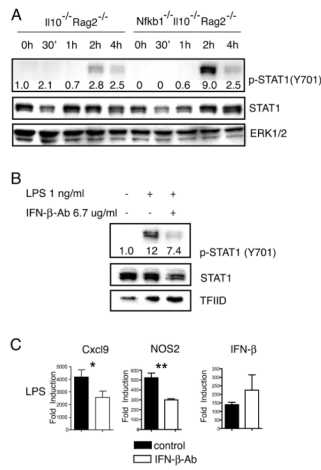


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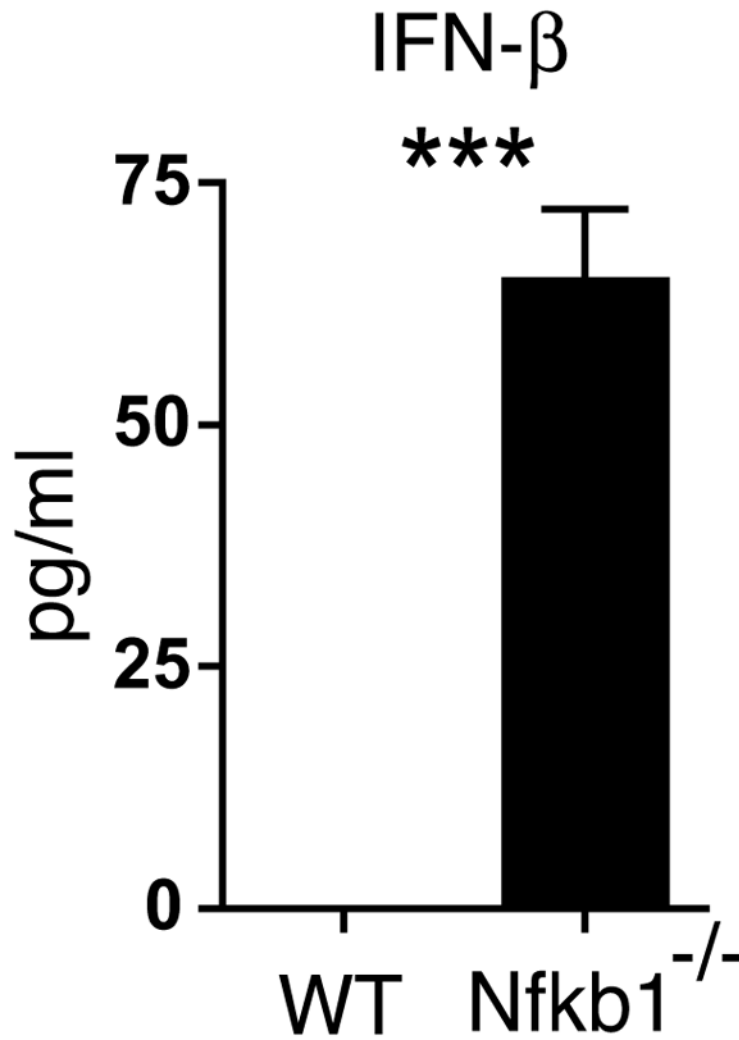
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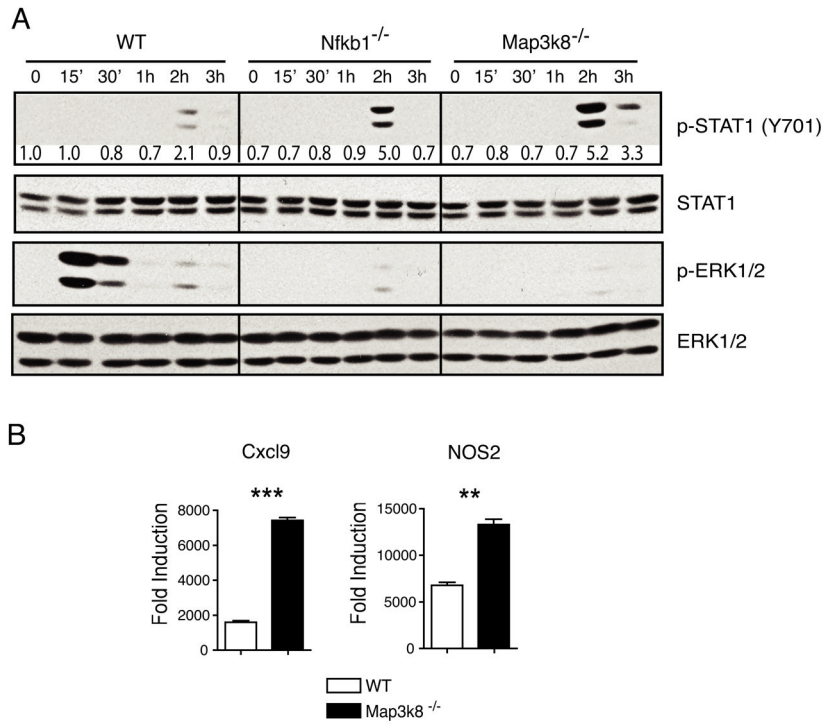
**Figure 1.** Effect of p50/p105 deficiency on LPS-induced gene expression. A) Fold-induction as determined by RT-PCR of IFN-responsive genes 4 hours after stimulation with LPS. B) Fold-induction of IFN non-responsive genes 4 hours after stimulation with LPS. C) Fold induction of indicated genes 4h after stimulation with IFN- $\beta$ . Please note log scale for y-axis. D) Fold-Induction of *Ifnb1* mRNA (left) or amount of IFN- $\beta$  protein accumulation in the medium as determined by ELISA (right), 4 and 24 hours after stimulation with LPS, respectively. Each column represents the mean of 3 independent macrophage pools for each genotype. One of two experiments with similar results.



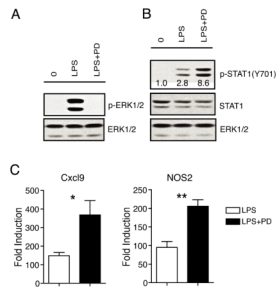
**Figure 2.** p50/p105-deficiency increases phosphorylation of STAT1 following TLR stimulation. A) Immunoblot of p-STAT1 (Y701) and total STAT1 in cell extracts from *Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* and *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM following stimulation with LPS at indicated time points. ERK is included as a loading control. Numbers under the p-STAT1 band indicate the intensity of the p-STAT1 band relative to the intensity in the unstimulated *Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* sample, normalized to total STAT1. B) Immunoblot of p-STAT1 (Y701) and total STAT1 in nuclear extracts from *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM 4h after stimulation with LPS in the presence or absence of IFN-β-specific depleting antibody. TFIID is included as a nuclear loading control. C) Fold induction of indicated IFN responsive genes in *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM 4h after stimulation with LPS in the presence or absence of an IFN-β blocking antibody. Columns represent mean values of 3 independent macrophages pools for each genotype. One of two experiments with similar results.



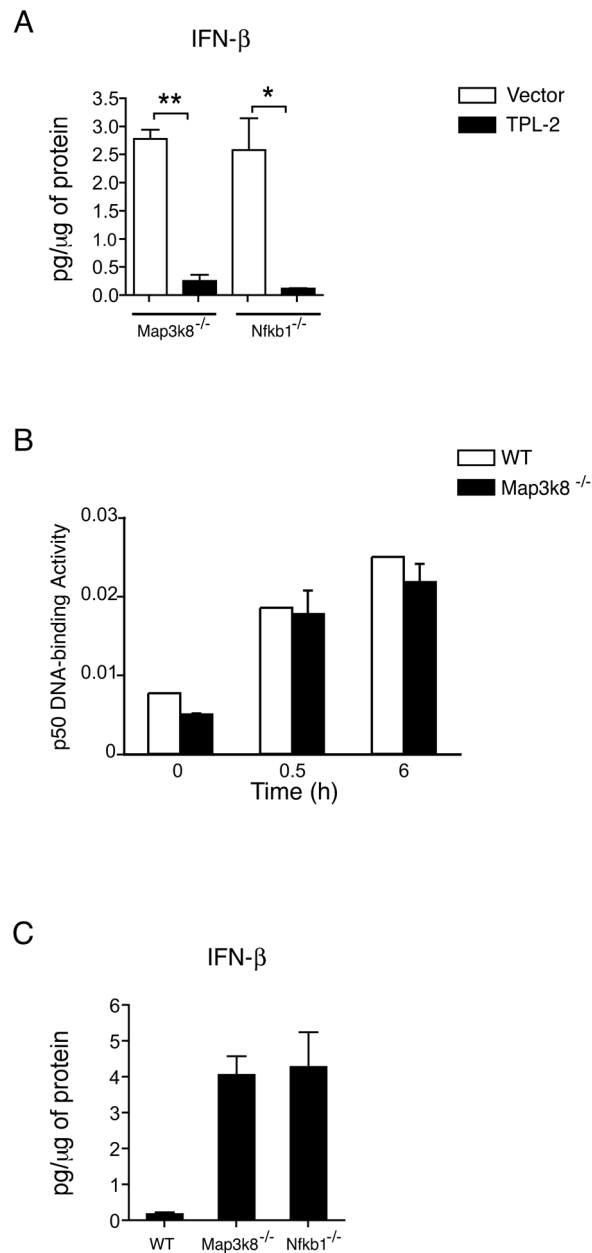
**Figure 3.** Increased levels of IFN- $\beta$  in the serum of *Nfkb1*<sup>-/-</sup> mice after LPS challenge. WT and *Nfkb1*<sup>-/-</sup> mice received 200  $\mu$ g of LPS and serum was harvested 4 hours later. IFN- $\beta$  levels were measured in the serum by ELISA. Each column represents 5 mice per genotype. One of two experiments with similar results.



**Figure 4.** Increased IFN-signaling in TPL-2-deficient BMDM. A) Immunoblotting of p-STAT1 (Y701), total STAT1, p-ERK 1/2, or total ERK in WT, *Nfkb1*<sup>-/-</sup>, or *Map3k8*<sup>-/-</sup> BMDM at indicated times following stimulation with LPS. B) Fold induction of *Cxcl9* and *Nos2* in WT or *Map3k8*<sup>-/-</sup> BMDM 4 hours after stimulation with LPS. Each column represents 2 independent mice per genotype. One of two experiments with similar results.

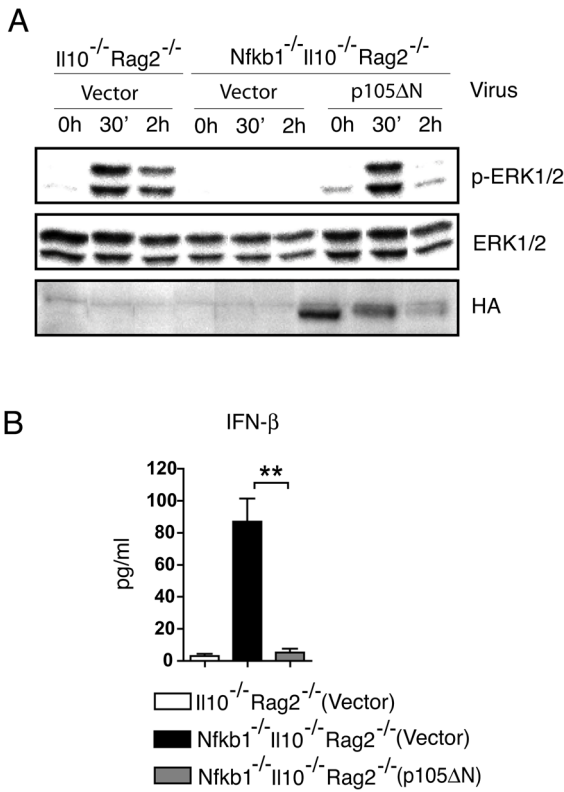


**Figure 5.** ERK inhibition augments LPS-induced IFN signaling. A) Immunoblotting of p-ERK and total ERK in total cell extracts of WT BMDM stimulated with LPS for 30 min ± PD184352 (PD). B) Immunoblotting of p-STAT1 (Y701), total STAT1, and total ERK in WT BMDM stimulated for 2h ± PD. C) Fold-induction of *Cxcl9* and *Nos2* mRNA in WT BMDM stimulated for 4h ± PD. Representative of three similar experiments.

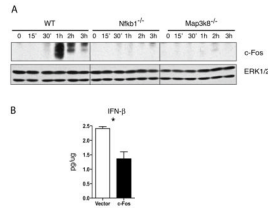


**Figure 6.** Expression of TPL-2 inhibits LPS-induced IFN- $\beta$  production by *Nfkb1*<sup>-/-</sup> macrophages. A) Accumulation of IFN- $\beta$  in medium 24 hours after LPS-stimulation of sorted GFP<sup>+</sup> *Map3k8*<sup>-/-</sup> and *Nfkb1*<sup>-/-</sup> BMDM, retrovirally infected with either control GFP vector (vector), or vector encoding GFP and TPL-2 (TPL-2). Levels of IFN- $\beta$  were measured by ELISA and normalized to total cellular protein. B) p50 DNA binding activity in WT or *Map3k8*<sup>-/-</sup> BMDM measured by NF- $\kappa$ B DNA binding ELISA. C) Accumulation of IFN- $\beta$  in the medium 24 hours after stimulation of WT, *Map3k8*<sup>-/-</sup>, and *Nfkb1*<sup>-/-</sup> BMDM with LPS. Each column represents mean of 2 mice. One of two experiments with similar results.





**Figure 7.** Expression of p105ΔN rescues ERK activation, and inhibits IFN-β secretion in *Nfkb1<sup>-/-</sup>* BMDM. A) *Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* and *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM infected with the vector alone (vector) or the vector encoding p105ΔN (p105ΔN) were lysed following stimulation with LPS, and immunoblotted for the indicated antigens. B) IFN-β secretion in culture supernatants was measured by ELISA 12 hours after LPS stimulation in *Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* and *Nfkb1<sup>-/-</sup>Il10<sup>-/-</sup>Rag2<sup>-/-</sup>* BMDM infected with the vector alone, or with p105ΔN. Each column represents the mean of 3 independent macrophage pools.



**Figure 8.** Expression of c-Fos down-regulates IFN- $\beta$  expression in *Nfkb1*<sup>-/-</sup> BMDM. A) Immunoblot of c-Fos in total cell extracts from WT, *Nfkb1*<sup>-/-</sup> and *Map3k8*<sup>-/-</sup> BMDM stimulated with LPS for indicated times. B) IFN- $\beta$  secretion following LPS stimulation of sorted GFP<sup>+</sup> *Nfkb1*<sup>-/-</sup> BMDM infected with the vector alone or infected with the vector encoding c-Fos. Each column represents 3 independent mice per group. One of 2 experiments with similar results.

**Table I**

Top 20 genes expressed at higher levels in *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup> BMDM than in *Il10*<sup>-/-</sup> BMDM following LPS stimulation<sup>1</sup>

Symbol	Name	FC	p
Gbp1	guanylate nucleotide binding protein 1	182	0.00
Iigp1	interferon inducible GTPase 1	19.0	0.01
Rsad2	radical S-adenosyl methionine domain containing 2	14.2	0.00
Mx1	myxovirus (influenza virus) resistance 1	14.0	0.01
Oas1l	2'-5' oligoadenylate synthetase-like 1	13.7	0.00
Tyki	thymidylate kinase family LPS-inducible member	12.6	0.01
Tgtp	T-cell specific GTPase	12.2	0.01
Slfn1	schlafen 1	12.2	0.02
Ifit2	interferon-induced protein with tetratricopeptide repeats 2	10.6	0.01
Mpa2l	macrophage activation 2 like	10.3	0.00
Il12b	interleukin 12b	10.2	0.00
Usp18	ubiquitin specific peptidase 18	9.96	0.01
Gfi1	growth factor independent 1	8.50	0.00
Il15	interleukin 15	7.24	0.00
Nt5c3	5'-nucleotidase, cytosolic III	7.01	0.00
Mx2	myxovirus (influenza virus) resistance 2	6.89	0.00
Iigp2	interferon inducible GTPase 2	6.29	0.00
Cxcl10	chemokine (C-X-C motif) ligand 10	6.06	0.00
Chi3l1	chitinase 3-like 1	5.82	0.00
Cxcl9	chemokine (C-X-C motif) ligand 9	5.75	0.01

<sup>1</sup>Fold Change (FC) refers to expression in *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup> BMDM divided by expression in *Il10*<sup>-/-</sup> BMDM following stimulation with LPS in the presence of exogenous IL-10. p refers to the P-value between genotypes calculated using the Benjamini-Hochberg correction for multiple comparison testing. All listed genes were induced at least 4 fold by LPS in either *Il10*<sup>-/-</sup> or *Nfkb1*<sup>-/-</sup>*Il10*<sup>-/-</sup> BMDM. Duplicates, Expressed Sequences Tags, and hypothetical genes have been removed.