Supporting Online Material for

Achieving Stability of Lipopolysaccharide-Induced NF-κB Activation

Markus W. Covert, Thomas H. Leung, Jahlionais E. Gaston, David Baltimore*

*To whom correspondence should be addressed. E-mail: baltimo@caltech.edu

Published 16 September 2005, *Science* **309**, 1854 (2005)
DOI: 10.1126/science.1112304

This PDF file includes:

Supporting Online Material

Achieving Stability of LPS-Induced NF-κB Activation

Markus W. Covert[*], Thomas H. Leung[*], Jahlionais E. Gaston and David Baltimore[‡]

Division of Biology

California Institute of Technology

Pasadena, CA 91125, USA

[*] These authors contributed equally to this work.

[‡] To whom correspondence should be addressed. Email: baltimo@caltech.edu

Materials and Methods

Cell lines and culture

Mouse embryo fibroblasts obtained from M. Yamamoto and S. Akira were grown in DMEM with 10% fetal calf serum. 100% confluent cells were treated for 48 hours with 0.5% serum containing medium and then stimulated with 0.5 μg/mL lipopolysaccharide (Sigma-Aldrich, St. Louis, MO) or 10 ng/mL murine TNFα (Roche, Palo Alto, CA). Where indicated, cells were pre-treated for 60 minutes with 25μg/mL cycloheximide (Sigma-Aldrich). Neutralizing antibodies and soluble receptors were obtained from R&D Systems, Inc. (Minneapolis, MN), and media concentrations were determined from the given ED_{50} range.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as described elsewhere (*S1*). Samples were normalized by Bradford assay. Every experiment was performed at least twice, most three times with high-reproducibility.

ELISA

After the cells were stimulated, 50 μL of medium was taken and analyzed according to the manufacturer's instructions (www.biosource.com).

RNA isolation and gene expression determination

Total RNA was isolated using a Qiagen RNEasy kit with on-column DNAse digestion (city, state), according to manufacturer's instructions (www.qiagen.com), and hybridized to Affymetrix Mouse Genome 430 2.0 Gene Chips, according to specified protocols (www.affymetrix.com). Rosetta Resolver (Rosetta Biosoftware, Seattle, WA) was used to normalize and analyze the chip data.

cDNA synthesis was performed according to manufacturer's instructions (www.appliedbiosystems.com). Q-PCR was performed using Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA) and an Applied Biosystems Real-Time PCR machine. Samples were performed in triplicate and GAPDH or β-actin transcript was used to normalize between samples. Every experiment was performed at least twice, many three times with high reproducibility. Primer sequences are available upon request.

siRNA The siRNA vectors and production were produced as described elsewhere (*S2*,*S3*).

IKK Assay

Anti-IKKγ immunoprecipitates (Santa Cruz Biotechnologies SC-8256) from WT, Trif-, and MyD88-deficient extracts stimulated with LPS for the indicated times were assayed for 30 minutes by a standard immune complex kinase assay. GST-IκBα (1-62) was used as a substrate. To ensure equal loading, Western analysis was performed using an anti-IKKα antibody (Imgenex IMG-136).

Computational Methods

We described a model used to describe the feedback control of NF-κB by IκBα previously (*1*). This model does not explicitly include upstream pathways, but was made to describe the response of immortalized mouse embryo fibroblasts to TNFα. This model was modified and expanded to enable simulation of the TLR-4 pathways to NF-κB activation, as described below. We used Berkeley Madonna software (Berkeley, CA) to perform all of the calculations described here.

Modifications

The parameters of oscillation observed in our specific cell type differed from those observed in our earlier study, and therefore we re-fit some of the parameters based on our own EMSA measurements of wild-type MEFs stimulated with TNFα as described above. The resulting parameter changes are shown in Table S1.

For all of the parameters, the changes are relatively minor and mostly serve to decrease the oscillatory period. In three cases (k1, tr1, tr2), no measurement has been made and the previous model also determined these parameters by a fit. In the case of tr3, one study determined a spontaneous half-life of ~30 minutes for IκB RNA transcripts. Our degradation rate roughly corresponds to a half-life of 15 minutes, and the model was comparatively sensitive to this parameter.

Expansion

To simulate the interactions between the MyD88-dependent and -independent pathways, we represented each pathway as a first-order process with parameters as shown in Figure 2C. The parameters were fit using Berkeley Madonna's curve fitting function. LPS stimulation was represented as a step input of 0.09. Model files are available upon request.

Figures

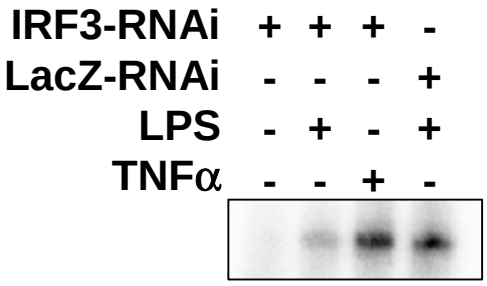

Figure S1. IRF-3 knockdown controls. (A) EMSA was used to determine activation of NF-κB in MyD88-deficient MEFs stimulated for 90 minutes with LPS or TNFα, where siRNA was inserted in a lentiviral vector to knockdown the expression of IRF-3 or LacZ

(*2*, *3*). The reduction in IRF-3 expression was approximately 4-fold, as confirmed by Western blot.

Tables

Table S1. Parameter changes made to the Hoffmann/Levchenko model (*S1*). "Old" represents the H/L parameter, while "New" represents the value used in this study.

Symbol [units]	***Old***	***New***	***Description***
k1 [-]	5.4	3.0	Nuclear NF-κB transport rate constant
tr1 [-]	0.2448	0.40	IκB translation rate constant
tr2 [-]	0.99	0.94	Enhancement of NF-κB on translation of IκBα
tr3 [1/min]	0.0168	0.08	Degradation rate of IκB transcripts

Table S2. Significant gene expression after 45 minutes of LPS stimulation as detected by Affymetrix microarray.

Gene Name	**Intensity Fold Change**	**P-value**
Scyb2/MIP-2	22.3	7.10E-42
TNFa	13.0	2.20E-20
Tnfsf9/4-1BB	7.1	8.30E-20
Scya4/MIP-1ß	6.5	6.30E-08
CD-83	6.4	1.00E-13
Scya3/MIP-1a	4.2	8.20E-15
Scyb10/IP-10	3.4	3.20E-04

References

S1. A. Hoffmann, A. Levchenko, M. L. Scott, D. Baltimore, *Science* 298, 1241 (Nov 8, 2002).

S2. T. H. Leung, A. Hoffmann, D. Baltimore, *Cell* 118, 453 (Aug 20, 2004).

S3. X. F. Qin, D. S. An, I. S. Chen, D. Baltimore, *Proc Natl Acad Sci U S A* 100, 183 (Jan 7, 2003).