Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock

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Activation of the mitogen-activated protein kinase (MAPK) cascade after Toll-like receptor stimulation enables innate immune cells to rapidly activate cytokine gene expression. A balanced response to signals of infectious danger requires that cellular activation is transient. Here, we identify the MAPK phosphatase dual specificity phosphatase 1 (DUSP1) as an essential endogenous regulator of the inflammatory response to lipopolysaccharide (LPS). DUSP1-deficient (DUSP1$^{-/-}$) bone marrow–derived macrophages showed selectively prolonged activation of p38 MAPK and increased cytokine production. Intraperitoneal challenge of DUSP1$^{-/-}$ mice with LPS caused increased lethality and overshooting production of interleukin (IL)-6 and tumor necrosis factor α. Transcriptional profiling revealed that DUSP1 controls a significant fraction of LPS-induced genes, which includes IL-6 and IL-10 as well as the chemokines CCL3, CCL4, and CXCL2. In contrast, the expression of the important mediators of endotoxin lethality, interferon γ and IL-12, was not significantly altered by the absence of DUSP1. These data together demonstrate a specific regulatory role of DUSP1 in controlling a subset of LPS-induced genes that determines the outcome of endotoxin shock.

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Innate immune cell activation via Toll-like receptors induces the release of inflammatory cytokines, chemokines, and mediators. A rapid and robust response of macrophages and dendritic cells is needed for the control and elimination of fast-growing microbial invaders. Nevertheless, the intensity and duration of this response have to be limited to avoid excessive inflammatory tissue damage. A range of regulatory factors acts at multiple levels to restrict or down-regulate the cytokines released from activated macrophages. These include the inhibitory cytokines IL-10 and TGF-β; cytokine antagonists, such as IL-1Rn and IL-18 binding protein; intracellular modulators of signaling pathways, such as the phosphatase SHIP (1); members of the suppressor of cytokine signaling protein family (2); and transcriptional regulators, such as c-maf (3) and Bcl-3 (4). Functionally, LPS-triggered activation of macrophages induces a state of refractoriness to further stimulation, a process termed LPS tolerance that is incompletely understood but may involve one or the other of the above-mentioned molecular players (5).

TLR signaling via Myd88 activates two major pathways linking the cell surface to transcriptional activation in the nucleus: besides release of Rel proteins from IκB for nuclear translocation, the mitogen-activated protein kinase (MAPK) cascade is activated and p38, ERK1/2, and JNK are all activated through phosphorylation by their respective upstream kinase (for review see reference 6). The termination of MAPK activity by endogenous regulators is evident from the transient kinetics of TLR-induced phosphorylation that, dependent on the cell type and experimental conditions used, returns to base levels after one to several hours. During the last decade, a family of dual specificity phosphatases (DUSPs) was identified that acts as MAPK phosphatases by binding to activated MAPKs and dephosphorylating them at threonine and tyrosine residues. The prototypic member of this family, DUSP1 (also known as mkp-1, erp, Ptpn16, and 3CH134),

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was identified as an immediate early gene in serum-stimulated fibroblast (7) but is expressed in many cell types in response to growth factors, stress, or heat shock (for review see reference 8). In macrophages, DUSP1 expression is increased upon stimulation with LPS or peptidoglycan (9, 10), but also by dexamethasone (11). We recently found that IL-10 increases DUSP1 expression in activated macrophages (12). Using DUSP1$^{-/-}$ embryonic fibroblasts and alveolar macrophages, it was shown that DUSP1 deficiency results in prolonged activation of p38 MAPK (11, 13).

We have used DUSP1$^{-/-}$ mice (14) to investigate the requirement of DUSP1 for regulation of the innate immune response to LPS in vivo. Our data indicate that DUSP1 regulates a subset of LPS-induced genes, whose overproduction in DUSP1$^{-/-}$ mice is sufficient to cause 100% lethality after injection of a normally sublethal dose of LPS. These findings point to a nonredundant role of DUSP1 in balancing innate immune responses and suggest more specific functions of the different DUSPs than previously anticipated.

RESULTS AND DISCUSSION

LPS-induced IL-6 production and MAPK activation in DUSP1$^{-/-}$ macrophages

Using primary bone marrow–derived macrophages from WT and DUSP1$^{-/-}$ mice, we observed significantly higher production of IL-6 in response to titrated amounts of LPS (Fig. 1 A). The secretion of TNF-α by LPS-stimulated macrophages was less affected by DUSP1 deficiency but higher than in WT (not depicted). These data confirm and extend the recently reported increase in TNF-α production in DUSP1$^{-/-}$ alveolar macrophages (11) and demonstrate differential control of various cytokines by DUSP1. In a kinetic analysis of MAPK phosphorylation, we found primarily an effect of DUSP1 deficiency on the down-regulation of p38 activation that led to markedly increased phospho-p38 levels at later time points (Fig. 1 B). In contrast, the kinetics of ERK1/2 activation were similar in WT and DUSP1$^{-/-}$ macrophages, whereas JNK showed a transiently increased phosphorylation status in the absence of DUSP1. Thus, DUSP1 is essential for the control of p38 activation in LPS-activated macrophages, which is consistent with earlier data showing selectivity of this MAPK phosphatase for p38 over ERK1/2 (15) and corroborates data by Zhao et al. (11) that demonstrate prolonged p38 activation in DUSP1$^{-/-}$ alveolar macrophages. In addition to DUSP1, LPS induces DUSP2, DUSP10, and DUSP16 in macrophages (12, 16, 17); apparently, however, these cannot fully compensate for a lack of DUSP1 in terms of p38 regulation, but may be more effective in regulating JNK and ERK1/2 activity.

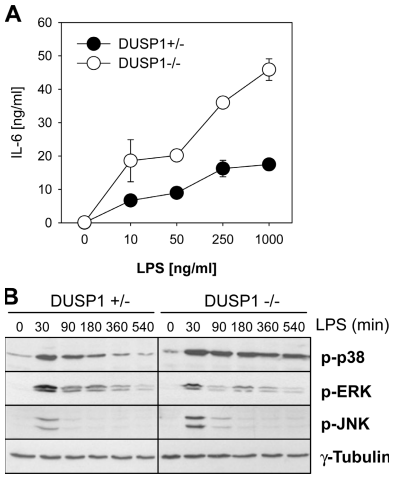

Figure 1. In vitro analysis of macrophages from DUSP1$^{-/-}$ mice. (A) Production of IL-6 from LPS-treated macrophages was analyzed by ELISA in supernatants harvested 8 h after stimulation. The mean and standard deviations of triplicate measurements from a representative experiment are shown. (B) Kinetics of MAPK activation by 100 ng/ml LPS in macrophages generated from bone marrow cells of DUSP1$^{+/-}$ or DUSP1$^{-/-}$ mice. 1.5 × 10^6 cells were stimulated for the indicated times, and whole cell lysates were analyzed by Western blot using antibodies to phosphorylated p38, ERK1/2, JNK, and to γ-tubulin at a 1:1,000 dilution. Data shown are from one representative experiment out of three performed.

Increased susceptibility of DUSP1$^{-/-}$ mice to lethal LPS shock

Based on these in vitro data, we asked whether DUSP1 determines the response to LPS in vivo. First, we observed that after i.p. injection of LPS, DUSP1 mRNA expression was induced rapidly in the spleen (Fig. 2 A), lung, and liver (not depicted). To test the hypothesis that induction of DUSP1 expression is required to limit the inflammatory response in a negative feedback loop, we used DUSP1$^{-/-}$ mice in a high-dose LPS shock model (Fig. 2 B). At an LPS dose of 25 mg/kg body weight, all the injected DUSP1$^{-/-}$ mice died between 20 and 48 h thereafter. In contrast, >90% of WT mice survived.

Cytokine production in vivo

Serum levels of cytokines with a known role in the pathogenesis of endotoxin shock were analyzed to investigate the mechanism underlying the high susceptibility to LPS in the absence of DUSP1 (Fig. 2 C). Already 1.5 h after injection of LPS, the levels of TNF-α and IL-6 were significantly elevated in DUSP1$^{-/-}$. At the later time points, differences were even more pronounced for IL-6, whereas TNF-α was down-regulated in the DUSP1$^{-/-}$, although still higher than in WT (Fig. 2 C). Because DUSP1$^{-/-}$ and WT mice were on a mixed background, we confirmed the differences in IL-6 and TNF-α levels 6 h after LPS injection in mice backcrossed on pure backgrounds to exclude confounding by modifier genes (Fig. 2 C, right). We also analyzed IFN-γ and IL-12p40, both known to contribute to lethal outcome of endotoxin shock (18–20), and found no significant effect of DUSP1 deficiency 6 h after injection (Fig. 2 D; not depicted for IFN-γ). In contrast, IL-10$^{-/-}$ mice that are also highly susceptible to LPS shock (21) exhibited uncontrolled

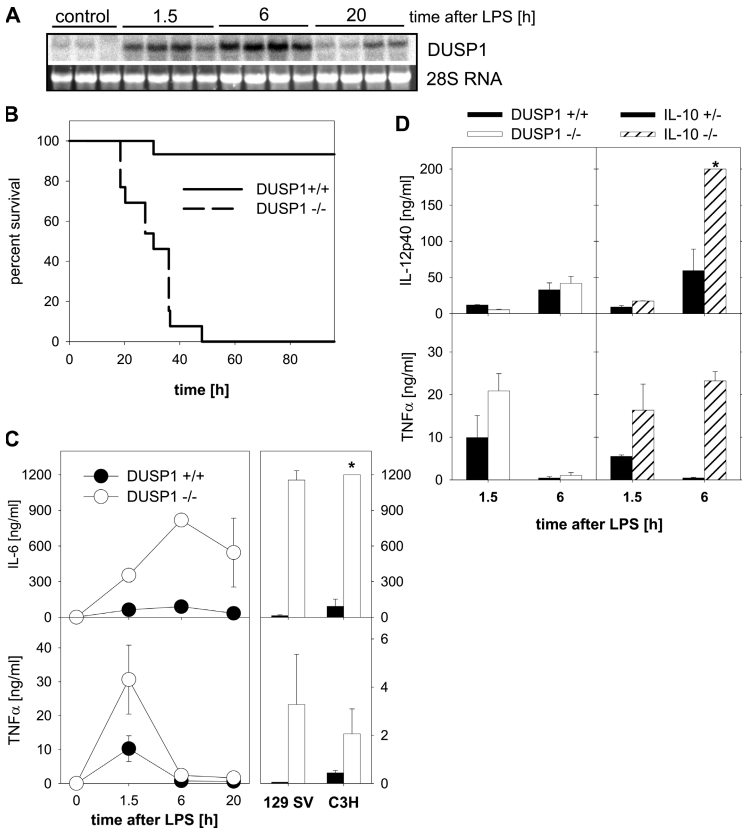

Figure 2. DUSP1 is induced after injection of LPS and controls survival and cytokine production. (A) Expression levels of DUSP1 in the spleens of WT mice were determined at the indicated times after injection of 10 mg/kg LPS i.p. by Northern blot analysis of 10 µg total RNA. Data shown are from individual mice (n = 3–4) from one representative experiment. (B) Survival of WT (n = 15) and DUSP1$^{-/-}$ mice (n = 13) after i.p. injection of 25 mg/kg LPS. Data are pooled from two experiments. (C and D) Serum levels of cytokines after LPS injection (10 mg/kg). Data shown are the mean and standard deviation (n = 3–4 mice per data point) from representative experiments. Two data points marked by asterisks were beyond the maximum of the standard curve, and the upper limits of the dynamic range of the assay are shown. (C) TNF-α and IL-6 in WT and DUSP1$^{-/-}$ mice on a mixed 129Sv × Bl/6 (left) or pure 129Sv and C3H (right) background (6 h after LPS). (D) Comparison of the effect of deficiency in DUSP1 or IL-10 on serum levels of TNF-α and IL-12p40.

release of IFN-γ, IL-12, IL-6, and TNF-α (Fig. 2 D and reference 21).

Genome-wide analysis of DUSP1-regulated gene expression in LPS-challenged mice

To obtain a global view of the impact DUSP1 has on LPS-induced gene expression, transcriptional profiling was performed using spleen RNA prepared 6 h after LPS challenge. In both groups of mice, LPS induced substantial changes in gene expression, with a considerable overlap in the genes induced in WT or DUSP1$^{-/-}$ mice (Fig. 3 A, Venn diagram; see Table S1, available at http://www.jem.org/cgi/content/full/jem.20051753/DC1, for a complete list of 608 up-regulated genes). However, in DUSP1$^{-/-}$ mice, nearly threefold more genes were uniquely up-regulated compared with WT, which is also evident from the hierarchical clustering analysis, with cluster C containing 229 genes induced more strongly in the absence of DUSP1 (Fig. 3 A; see also box plots for the various clusters in Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051753/DC1). The microarray data corroborate most of the cytokine data obtained by ELISA from serum samples. IL-6 was up-regulated in DUSP1$^{-/-}$ mice, whereas IL-12p40 and IFN-γ showed no difference in expression. A notable exception is TNF-α, whose expression was not increased in DUSP1$^{-/-}$ mice at the mRNA level, a result that was confirmed by Northern analysis (Fig. S2) and likely due to posttranscriptional effects (e.g., on mRNA stability or translation).

Control of selected inflammatory molecules by DUSP1

We mined the microarray data with regard to the question of how DUSP1 deficiency causes excess lethality in LPS challenge. Although some downstream effectors of LPS-induced lethality may not yet be expressed at the relatively early time point analyzed in this experiment, DUSP1$^{-/-}$ mice already appeared sicker clinically. Therefore, it seems reasonable to assume a contribution to the severe inflammatory response for at least some of the genes overexpressed in the absence of DUSP1. Among the cytokines that have been implicated as

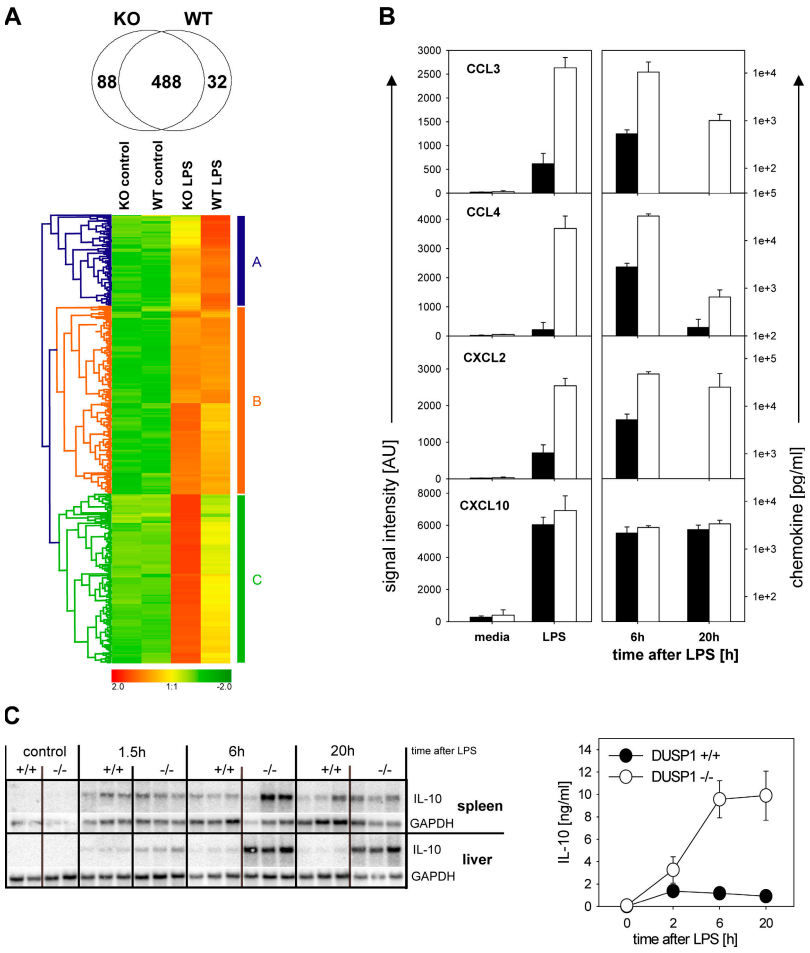

Figure 3. Genome-wide analysis of LPS-induced gene expression in the spleens of WT and DUSP1$^{-/-}$ mice. (A) Total splenic RNA was prepared from control WT and DUSP1$^{-/-}$ mice 6 h after i.p. injection of 10 mg/kg LPS (n = 3 per group) and was processed for Affymetrix GeneChip analysis. Venn diagram comparing the numbers of genes significantly up-regulated in WT and DUSP1$^{-/-}$ mice by LPS (for criteria see Materials and methods). Z-score–normalized expression values of LPS-induced genes were subjected to hierarchical clustering analysis. Cluster A contains a group of genes expressed more strongly in the WT spleen. In contrast, cluster C represents a larger subset of LPS-induced genes up-regulated in the absence of DUSP1. Expression data of these 608 genes can be found in Table S1. (B) Differential control of the chemokines CCL3, CCL4, CXCl2, and CXCL10 by DUSP1. Confirmation of the microarray expression data (left: average and standard deviation of signal intensity values) by ELISA determination of serum levels (right: mean and standard deviation, n = 3–4). (C) Increased expression of IL-10 at the mRNA and protein level in DUSP1$^{-/-}$ mice. Northern data from two and three individual mice per condition and organ are shown, and ELISA data are the mean and standard deviation (n = 3–4) from a representative experiment.

contributors to the pathogenesis of lethal endotoxin shock, IFNs type I and II as well as IL-12, TNF- α, IL-1α, and IL-18 were expressed at similar levels in WT and DUSP1$^{-/-}$ spleen (Table S2). On the other hand, the chemokines CCL3, CCL4, and CXCL2 were among the LPS targets overexpressed most strongly in DUSP1$^{-/-}$ spleens, a finding that was validated at the protein level by corresponding serum levels (Fig. 3 B), and may contribute to the development of shock. For example, CCL3 (MIP-1α) promotes leukocyte recruitment to the lungs and increases lethality (22), an effect that may be enhanced by the closely related CCL4 (MIP-1β) and CXCL2 (MIP-2). In contrast, CXCL10 (IP-10) was not affected by the absence of DUSP1 at the mRNA or protein level (Fig. 3 B).

The production of IL-10 is regulated by DUSP1

The microarray analysis identified the immunoregulatory cytokine IL-10 to be induced 3.3-fold stronger in the DUSP1$^{-/-}$ spleen, which was confirmed by Northern blotting (Fig. 3 C). This difference was strikingly more pronounced in the liver, which may account for the 10-fold higher serum IL-10 levels in DUSP1$^{-/-}$ compared with WT mice 6 and 20 h after LPS challenge (Fig. 3 C). Higher production of IL-10 in response to LPS was also observed in DUSP1$^{-/-}$ macrophages stimulated in vitro (not depicted). Among the genes overexpressed in DUSP1$^{-/-}$ spleens, we observed a considerable number of transcripts previously identified as IL-10–induced genes (23), including SOCS3, NFIL3, Ndr1, and Gadd45γ (Table S1), probably reflecting secondary effects of

the overshooting production of IL-10 and IL-6 that both activate transcription via Stat3 signaling. However, high IL-10 levels in DUSP1$^{-/-}$ mice were not effective in down-regulating synthesis of IL-6 or the chemokines CCL3, CCL4, or CXCL2 (Fig. 3 B). Thus, DUSP1 may be required for inhibition of at least some IL-10–regulated genes.

Concluding remarks

The finding of a nonredundant role for DUSP1 in balancing the inflammatory response suggests that the various members of the DUSP family of MAPK phosphatases fulfill specific regulatory roles in innate immune cells. The recent report of increased JNK activation in innate and adaptive immune cells in the absence of DUSP10 is another example of this apparent division of labor between different MAPK phosphatases (17). In the case of DUSP1, the p38 MAPK pathway likely is the major target, and DUSP1 deficiency unleashes the expression of a rather selective set of TLR-induced genes that can be hypothesized to be direct or indirect targets of p38 MAPK. However, it is also possible that in addition to the various MAPKs, DUSP1 dephosphorylates other substrates as well. Surprisingly, the lethal outcome of LPS challenge in DUSP1$^{-/-}$ is correlated with excessive production of the anti-inflammatory cytokine IL-10. This counterintuitive finding may indicate that important inhibitory effects of IL-10 on inflammation depend on DUSP1 function. Similarly, in the myeloid-specific Stat3-deficient mouse, abrogated IL-10 signaling leads to overshooting, lethal cytokine production that includes IL-10 (24). Given the phenotype of DUSP1$^{-/-}$ mice in the high-dose LPS model reported here, it will be interesting to investigate the contribution of this phosphatase to the host response in models of infection and polymicrobial sepsis. Finally, the DUSP family emerges as a potential target for immunomodulation; identification of small molecule inhibitors has recently been reported (25, 26) and holds promise for selective therapeutic intervention.

MATERIALS AND METHODS

Mice, cells, and reagents. DUSP1$^{-/-}$ blastocysts generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute (14) were supplied by The Jackson Laboratory and subsequently bred on a mixed 129Sv × C57BL/6 background at the Forschungszentrum Karlsruhe. All experiments were performed according to European and German statutory regulations and approved by the Regierung von Oberbayern. The genotype of the mice was established by tissue biopsies and subsequent DNA analysis by PCR using two separate reactions with the allele-specific primers 5′-CAGGTACTGTGTGTCGGTGGTGCTAATG-3′ (WT) and 5′-AAATGTGTCAGTTTCATAGCCTGAAGAACG-3′ (mutant), which were used with the common reverse primer 5′-CTATATCCTCCTGGCACAATCCTCCTAG-3′, respectively.

For control experiments, mice backcrossed for seven generations onto C3H and 129Sv were used. Mice were used for LPS challenge at an age of 5–10 wk. Experimental groups were matched for age and sex. *Escherichia coli* O55:B5 LPS (no. L2880; Sigma-Aldrich) was diluted in sterile PBS and injected i.p. Bone marrow–derived macrophages were differentiated for 5–7 d in M-CSF containing media as described previously (27).

Detection of cytokines by ELISA. Serum and cell culture supernatants were analyzed for cytokine content using Duoset antibody pairs (R&D Systems) for detection of IL-6, TNF-α, IFN-γ, CXCL10, and IL-10. IL-12p40 production was measured with an OptiEIA kit from BD Biosciences, and CXCL2, CCL3, and CCL4 were measured with Quantikine kits from R&D Systems.

Analysis of MAPK activation by immunoblotting. Bone marrow–derived macrophages were treated as indicated and processed for analysis by Western blot as described previously (27). Antibodies for phosphorylated and total p38, JNK, and ERK1/2 were from Cell Signaling, and anti–γ-tubulin antibody was from Sigma-Aldrich and used at a 1:1,000 dilution.

Microarray analysis of gene expression. Total spleen RNA (5 μg) was labeled and hybridized to Affymetrix MOE430A 2.0 GeneChips according to the manufacturer's instructions. Three biological replicates per condition were analyzed. CEL files were processed for global normalization and generation of expression values using the rma algorithm in the R affy package (http://www.bioconductor.org). Normalized CEL expression values deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) as series GSE3565.

The list of significantly regulated genes was achieved by applying the SAM multiclass algorithm (28) of the samr package for R (FDR < 1%: 1,465 probe sets). Further filtering included a minimum fold-change criterion between all four experimental conditions of ±2 (1,372 probe sets) and a max (all mean expression values) − min (all mean expression values) filter of >50 (1,215 probe sets). Of these, 608 were up-regulated. Further data preparation was performed with the Spotfire DecisionSite software (Spotfire), and hierarchical clustering was performed using the program Genesis (release 1.1.3; reference 29).

Northern blot analysis of mRNA expression. Northern blot analysis was performed as described previously (23) using cDNA probes obtained from Deutsches Ressourcenzentrum für Genomforschung.

Online supplemental material. A list of all 608 genes that were found up-regulated by microarray analysis in WT or DUSP1$^{-/-}$ spleen 6 h after LPS injection can be found in Table S1. The expression levels of cytokines, chemokines, and ILs, as well as their receptors, in this microarray dataset are depicted in Table S2. Marked in bold are the probe sets showing significant differences in expression between WT and DUSP1$^{-/-}$ mice after LPS challenge with a p-value of <0.01. Fig. S1 shows box plots complementing the microarray data from Fig. 3 A. Fig. S2 contains Northern blot validations of microarray results. Supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051753/DC1.

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