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IRF3 Mediates a TLR3/TLR4-Specific Antiviral Gene Program

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Summary

We have identified a subset of genes that is specifically induced by stimulation of TLR3 or TLR4 but not by TLR2 or TLR9. Further gene expression analyses established that upregulation of several primary response genes was dependent on NF-κB, commonly activated by several TLRs, and interferon regulatory factor 3 (IRF3), which was found to confer TLR3/TLR4 specificity. Also identified was a group of secondary response genes which are part of an autocrine/paracrine loop activated by the primary response gene product, interferon β (IFNβ). Selective activation of the TLR3/TLR4-IRF3 pathway potently inhibited viral replication. These results suggest that TLR3 and TLR4 have evolutionarily diverged from other TLRs to activate IRF3, which mediates a specific gene program responsible for innate antiviral responses.

Introduction

Challenge by invading pathogens has led multicellular organisms to develop a number of defensive measures for the recognition and clearance of infectious agents. The innate immune system is capable of recognizing a wide variety of pathogens and rapidly induces a number of antimicrobial and inflammatory responses. Toll-like receptors (TLR) play a critical role in innate immunity by recognizing structurally conserved bacterial and viral components termed pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1998). Ten TLRs have been cloned in mammals, and each receptor appears to be involved in the recognition of a unique set of PAMPs. While the focus of many studies has been mainly on bacterial components, TLR3, TLR4, and TLR7 have been shown to mediate the response to the viral-associated PAMPs: the double-stranded RNA analog poly I:C; the F protein of Respiratory Syncytial Virus (RSV); and the antiviral therapeutic compounds, the imidazoquinolines, respectively (Alexopoulou et al., 2001; Hemmi et al., 2002; Kopp and Medzhitov, 1999; Kurt-Jones et al., 2000; Takeuchi and Akira, 2001; Takeuchi et al., 1999).

TLRs activate signaling through the Toll/IL-1R (TIR) domain found in the cytoplasmic tails of these proteins (Akira, 2000; Akira et al., 2001; Guha and Mackman, 2001; Takeuchi and Akira, 2001). Receptor activation triggers binding of the adaptor protein MyD88 (myeloid differentiation factor 88) to the TIR domain, allowing for interaction and autophosphorylation of IRAK (IL-1R-associated kinase) and subsequent activation of tumor necrosis factor receptor-associated factor 6 (TRAF6), leading to the activation of the NF-κB, JNK, PI3K, p38, and ERK pathways (Takeuchi and Akira, 2001; Ardeshna et al., 2000).

While all TLRs originally appeared to activate the same signaling pathways to initiate the inflammatory response, recent studies have indicated that the functional roles of TLR3 and TLR4 are more complex for several reasons. First, TLR4 has been shown to mediate the response to a wide variety of ligands other than lipopolysaccharide (LPS), including Gram-positive lipoteichoic acids, the cancer chemotherapeutic Taxol, and the F protein of RSV (Kurt-Jones et al., 2000; Medzhitov and Janeway, 1998; Takeuchi and Akira, 2001; Takeuchi et al., 1999). More perplexing is the fact that TLR4−/− mice have been shown to have increased susceptibility to infection by RSV, while no such finding has yet been reported in models of bacterial infection (Haynes et al., 2001). Also, TLR3 and TLR4 have been shown to activate NF-κB in cells lacking MyD88, albeit with delayed kinetics (Alexopoulou et al., 2001; Horng et al., 2001; Kawai et al., 1999). Recent reports indicate that the newly cloned TIR domain-containing molecule TIRAP/Mal may function as a second adaptor for TLR3 and TLR4 and direct activation of downstream signaling molecules in the absence of MyD88 (Fitzgerald et al., 2001; Horng et al., 2001).

Importantly, recent reports have described a role for interferon regulatory factor 3 (IRF3) in the TLR4 signaling cascade (Kawai et al., 2001; Navarro and David, 1999). IRF3 is an important transcriptional regulator of the antiviral immune response. Through an unknown mechanism, viral infection causes IRF3 to become phosphorylated and migrate to the nucleus where it participates in the activation of a complex positive feedback loop between Type I IFNs and IRF family members, leading to induction of an antigrowth, antiviral response (Sato et al., 2001; Taniguchi et al., 2001; Taniguchi and Takaoka, 2002). TLR4-mediated nuclear translocation of IRF3 has been shown to occur in a MyD88-independent fashion and to induce binding to interferon-stimulated response elements (ISRE) in vitro at 2 hr poststimulation (Kawai et al., 2001). However, the functional role of IRF3 in

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TLR3- or TLR4-induced gene expression remains largely undetermined.

Interestingly, members of the tumor necrosis factor receptor (TNFR) family use pathways similar to those utilized by TLRs to mediate quite different biological effects. One TNFR, CD40, has been shown to be intimately involved in the adaptive immune response (Foy et al., 1996). At the molecular level, we have found that CD40 stimulation activates the NF-κB, JNK, p38, and PI3K pathways (our unpublished data), while, functionally, CD40 is required for germinal center formation and affinity maturation (Gordon and Pound, 2000). Currently, the molecular mechanisms that differentiate TLR-mediated innate and TNFR-mediated adaptive immune responses are unknown.

To understand the signaling specificities between the TLR and TNFR families, we used Genechip microarray technology and compared the gene expression profiles of B-lymphocytes stimulated with CD40L and LPS. In this report, we describe a set of genes that is specifically induced by the activation of TLR3 or TLR4 but not TLR2, TLR9, or CD40. These genes, many of which have been previously described as viral or interferon-inducible, were further classified as primary or secondary response genes. We demonstrate that the primary response genes are coregulated by the NF-κB pathway, which is common for both TLRs and TNFRs, and the IRF3 pathway, which is responsible for TLR3/TLR4-specific gene activation. In addition, we show that several secondary response genes important for host defense are activated by autocrine/paracrine secretion of IFNβ. Activation of the TLR3/TLR4-IRF3 pathway potently inhibits the replication of murine γherpesvirus 68 (MHV68), demonstrating the functional significance of this pathway in antiviral responses. Overall, we have described a signaling and gene expression network specific to TLR3 and TLR4, and we provide evidence that these receptors play a unique role in the early detection and inhibition of viral infection by the innate immune system.

Results

LPS Induces a Subset of Genes Previously Characterized as "Interferon Regulated"

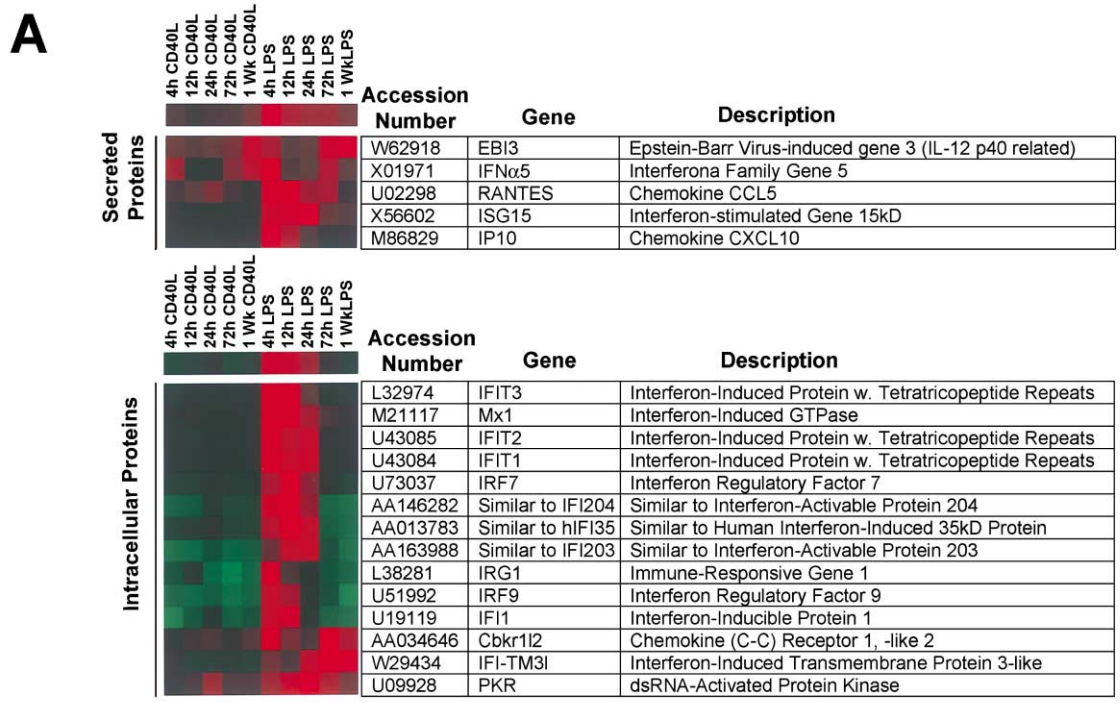
We have conducted a series of microarray experiments to determine gene expression patterns in murine B cells in response to activating stimuli such as LPS and CD40L. While CD40L specifically upregulates genes involved in cell-cell communication and germinal center formation (Dadgostar et al., 2002), hierarchical clustering and filtering of the microarray data also revealed a set of genes specifically induced by LPS, at least 19 of which have been previously classified as "interferon regulated." Figure 1A depicts a partial list of LPS-specific genes using color-based gene expression changes of Affymetrix probe sets with matching accession numbers, gene names, and descriptions. For a more complete analysis of microarray data, see Supplemental Data at http://www.immunity.com/cgi/content/full/17/3/251/DC1. Genes were hierarchically clustered using average difference change values derived by comparing control samples (media 4 hr) with samples from cells treated with indicated stimulus. Line charts of selected genes (Figure 1B) demonstrate similarities in kinetics of induction and LPS specificity. Microarray studies on bone marrow-derived macrophages (BMMs) established that IFNβ mRNA was also specifically upregulated by LPS at 2 hr (S.D. and G.C., unpublished data).

LPS-Primary Response Genes Exhibit TLR3/TLR4 Specificity

In order to understand the mechanism of selective gene activation by LPS, we conducted both Northern blot analysis and quantitative real-time PCR (Q-PCR), focusing on the macrophage cell type. As an example of our quantification methods, *RANTES* gene induction is shown using Northern blot analysis (Figure 2A) and Q-PCR (Figure 2B), which is used in all subsequent experiments. Throughout this report, the use of equivalent amounts of template in all Q-PCR reactions was controlled for by measurement of 18S rRNA amplification, except where noted. Cycloheximide treatment indicated that *RANTES* (Figures 2A and 2B), *IP10*, *IFNβ*, *IFIT1*, and *ISG15* (data not shown) induction is the direct result of primary signal transduction and did not require new protein synthesis. Similar results were seen in B cells (data not shown).

We then investigated the TLR specificity of gene expression in BMMs using specific agonists for TLR4 (lipid A), TLR2 (peptioglycan), TLR3 (poly I:C), and TLR9 (CpG). In order to account for differences in binding affinity and receptor expression, the concentrations of TLR ligands used for stimulation were titrated to produce roughly equivalent activation of the JNK pathway as determined by GST-c-Jun in vitro kinase assay (Figure 2C, upper left). Activation of NF-κB and the production of inflammatory cytokines are well-described for all known TLRs, and we found that our panel of TLR ligands induced both *IκBα*, a direct target of the NF-κB signaling pathway, and the inflammatory cytokine *TNFα* (Figure 2C, middle left, lower left). However, only TLR3 or TLR4 stimulation led to the immediate early upregulation of *IFNβ*, *IP10*, and *RANTES*, while minimal gene induction was observed with TLR2- or TLR9-agonists (Figure 2C, right panels). Interestingly, *IFNβ* was induced more potently by TLR3 than TLR4, while the chemokines *IP10* and *RANTES* were induced to roughly equivalent levels by stimulation of either receptor. No gene induction was observed in response to lipid A in TLR4 null BMMs generated from C57BL/10ScCr mice that carry a null mutation in the *TLR4* gene (Qureshi et al., 1999) (data not shown).

A summary of five TLR3/TLR4-specific primary response genes—*IP10*, *RANTES*, *IFNβ*, *ISG15*, and *IFIT1*—is shown in Figure 2D. These genes have been studied by other groups primarily in the context of viral infection and interferon stimulation (*IP-10* [Cole et al., 2001; Ohmori and Hamilton, 1993; Proost et al., 2001]; *RANTES* [Lin et al., 1999a; Luther and Cyster, 2001; Wagner et al., 1998]; *IFNβ* [Taniguchi and Takaoka, 2002]; *ISG15* [D'Cunha et al., 1996]; *IFIT1* [Guo and Sen, 2000; Smith and Herschman, 1996]). To identify common elements that might mediate TLR3/TLR4-specific gene induction, we analyzed the gene promoters using the 5′ 1 kb sequence obtained from Celera proprietary murine genomic databases and TESS promoter analysis software (http://www.cbil.upenn.edu/tess/). The regulatory



Accession Number	Gene	Description
W62918	EBI3	Epstein-Barr Virus-induced gene 3 (IL-12 p40 related)
X01971	IFNα5	Interferona Family Gene 5
U02298	RANTES	Chemokine CCL5
X56602	ISG15	Interferon-stimulated Gene 15kD
M86829	IP10	Chemokine CXCL10

Accession Number	Gene	Description
L32974	IFIT3	Interferon-Induced Protein w. Tetratricopeptide Repeats
M21117	Mx1	Interferon-Induced GTPase
U43085	IFIT2	Interferon-Induced Protein w. Tetratricopeptide Repeats
U43084	IFIT1	Interferon-Induced Protein w. Tetratricopeptide Repeats
U73037	IRF7	Interferon Regulatory Factor 7
AA146282	Similar to IFI204	Similar to Interferon-Activable Protein 204
AA013783	Similar to hIFI35	Similar to Human Interferon-Induced 35kD Protein
AA163988	Similar to IFI203	Similar to Interferon-Activable Protein 203
L38281	IRG1	Immune-Responsive Gene 1
U51992	IRF9	Interferon Regulatory Factor 9
U19119	IFI1	Interferon-Inducible Protein 1
AA034646	Cbkr1l2	Chemokine (C-C) Receptor 1, -like 2
W29434	IFI-TM3l	Interferon-Induced Transmembrane Protein 3-like
U09928	PKR	dsRNA-Activated Protein Kinase

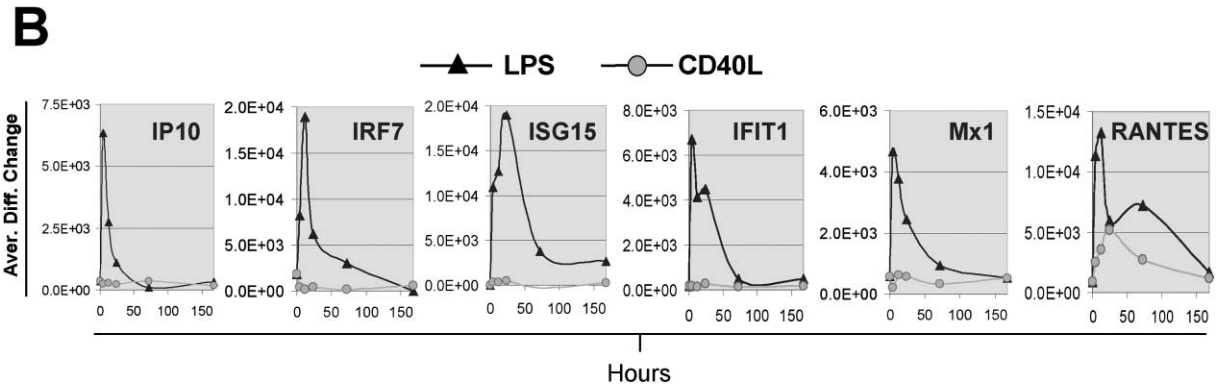


Figure 1. LPS but Not CD40L Upregulates a Set of Genes Previously Characterized as Interferon Responsive

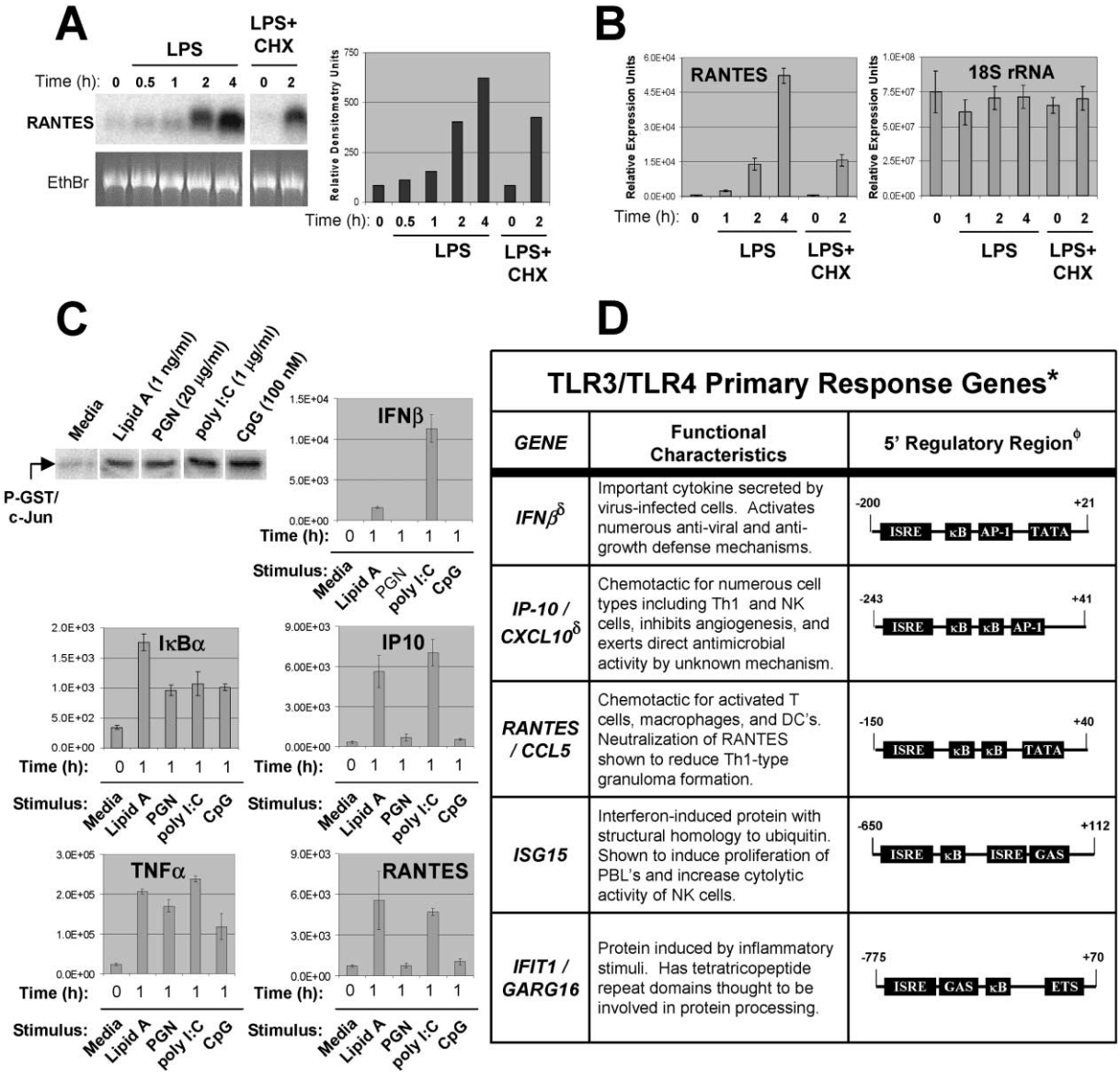
(A) Primary murine B cells were stimulated with LPS (20 μg/ml) or a soluble CD8/CD40L chimera (300 ng/ml), and RNA was collected at indicated time points and used to conduct microarray analysis. Genes previous characterized as interferon responsive were further subdivided, and absolute expression changes were displayed using the Treeview Program. Expression changes: red, induced; green, repressed; black, no change.

(B) Line charts display temporal expression pattern of selected genes from (A).

regions of all five genes showed high probability matches for ISRE and κB consensus sequences (Max. $lg[t_d] = 28.0$) within a few hundred base pairs of the transcriptional start site (Figure 2D). This indicated to us that these genes may be coregulated by common activators which bind at these sites.

IRF3 and NF-κB Are Involved in TLR3/TLR4-Mediated Gene Activation

Other groups studying models of viral infection have demonstrated binding of IRF3 to the ISRE consensus motifs in the promoters of *IFNβ* and *RANTES* (Lin et al., 1999a; Wathelet et al., 1999). While LPS treatment can induce the nuclear translocation of IRF3 and induce ISRE binding in vitro at 2 hr of stimulation (Kawai et al., 2001; Navarro and David, 1999), it was recently reported that LPS does not increase IRF3 transactivational activity (Servant et al., 2001). As a result, the role of IRF3 in response to PAMP-induced gene expression remains in question. Our promoter analyses led us to investigate the activation of IRF3 and NF-κB by TLR stimuli, as these transcription factors bind to ISRE and κB consensus sites, respectively. We first confirmed that TLR3- and TLR4-agonists but not TLR2- or TLR9-agonists induced rapid nuclear translocation of IRF3 (Figure 3A). However, unlike other reports, we found IRF3 to be activated within 15–30 min of treatment and to be insensitive to cycloheximide treatment (data not shown). In addition, stimulation of TLR3 could induce faster and more potent activation of IRF3 than TLR4, indicating further



TLR3/TLR4 Primary Response Genes*

GENE	Functional Characteristics	5' Regulatory Region$^{\phi}$
IFNβ$^{\delta}$	Important cytokine secreted by virus-infected cells. Activates numerous anti-viral and anti-growth defense mechanisms.	-200 ISRE κB AP-1 TATA +21
IP-10 / CXCL10$^{\delta}$	Chemotactic for numerous cell types including Th1 and NK cells, inhibits angiogenesis, and exerts direct antimicrobial activity by unknown mechanism.	-243 ISRE κB κB AP-1 +41
RANTES / CCL5	Chemotactic for activated T cells, macrophages, and DC's. Neutralization of RANTES shown to reduce Th1-type granuloma formation.	-150 ISRE κB κB TATA +40
ISG15	Interferon-induced protein with structural homology to ubiquitin. Shown to induce proliferation of PBL's and increase cytolytic activity of NK cells.	-650 ISRE κB ISRE GAS +112
IFIT1 / GARG16	Protein induced by inflammatory stimuli. Has tetratricopeptide repeat domains thought to be involved in protein processing.	-775 ISRE GAS κB ETS +70

Figure 2. Characterization of TLR3/TLR4-Primary Response Genes

(A) RAW 264.7 macrophages were stimulated with LPS (100 ng/ml) for the indicated time points, and RNA was harvested and analyzed by Northern blotting (left) using a cDNA probe for *RANTES*. CHX indicates 30 min pretreatment and costimulation with cycloheximide (20 μg/ml). Quantification of radioactive signal is depicted (right).

(B) Primary murine BMMs were stimulated with LPS (100 ng/ml) for the indicated time points, and RNA was harvested and then analyzed by quantitative real-time PCR (Q-PCR) for *RANTES* (left) and *18S* rRNA (right) expression. Experiments were conducted in triplicate. All Q-PCR data in this report are represented as relative expression units unless otherwise indicated.

(C) BMMs were stimulated with the following TLR-agonists: lipid A (1 ng/ml), peptidoglycan (PGN) (20 μg/ml), poly I:C (1 μg/ml), or CpG (100 nM) for 30 min, and cell extracts were used for an in vitro kinase assay using GST-c-Jun as a substrate (upper left). Identical stimulations were repeated for 1 hr, and RNA was harvested and used for Q-PCR analysis.

(D) Summary of TLR3/TLR4-primary response genes (see text for details).*, primary-reponse defined as upregulated by LPS (100 ng/ml) at 2 hr in the presence of cycloheximide (20 μg/ml). $^{\phi}$, schematic representation of gene promoters created using Celera web-based murine genomic database to obtain 5′ regulatory region, followed by theoretical analysis using TESS promoter analysis software (http://www.cbil.upenn.edu/tess/) and the TRANSFAC transcription factor database. Relevant consensus sequence matches are in accordance with published literature. $^{\delta}$, induced synergistically by LPS and CHX. DC, dendritic cells; PBL, peripheral blood leukocytes.

functional divergence between these two receptors. Similar results were seen with 1 μg/ml poly I:C (data not shown). In contrast, we observed nuclear translocation of p65 in response to all TLR-agonists tested in BMMs (Figure 3A) and RAW 264.7 macrophages (data not shown). Purity of cellular fractions was monitored by immunoblotting nuclear and cytoplasmic fractions for the resident proteins USF2 and tubulin, respectively (Figure 3B).

To determine whether IRF3 and NF-κB were involved in the LPS-induced transcriptional activity, we conducted chloramphenicol acetyl transferase (CAT) re-

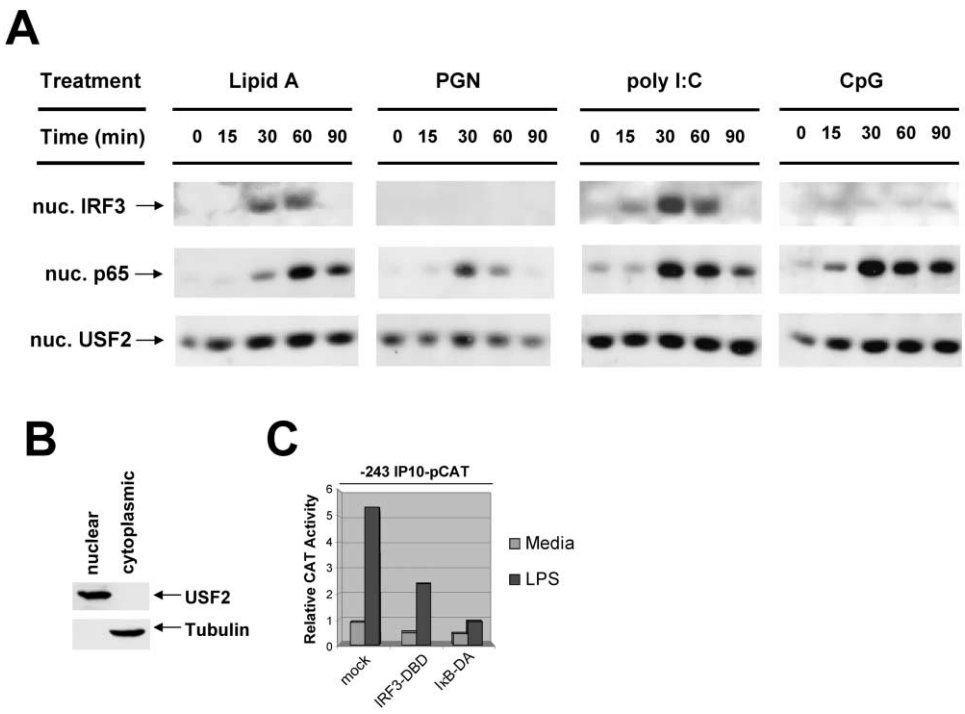


Figure 3. IRF3 and NF-κB Are Involved in TLR3/TLR4-Mediated Gene Activation

(A) BMMs were treated for indicated time points with lipid A (1 ng/ml), peptidoglycan (PGN) (20 μg/ml), poly I:C (10 μg/ml), or CpG (100 nM). Cells were fractionated, and 30 μg of nuclear extract was analyzed by SDS-PAGE immunoblotting for IRF3 nuclear translocation followed by stripping and reprobing for p65 and USF2.

(B) Purity of cellular fractionation was tested by immunoblotting for USF2 and tubulin.

(C) CAT reporter assay showing LPS-induced transactivation of the IP10 promoter. RAW 264.7 macrophages were transiently transfected with 1 μg of −243-IP10-pCAT and cotransfected with 6 μg of pCDNA3 (mock), pEBB-IRF3-DBD, or pCDNA3-IκBm-ER (IκB-DA). Six hours posttransfection, cells were treated with media or LPS (100 ng/ml) for 24 hr, and 30 μg of protein was used for each CAT reaction. Results are representative of three separate experiments.

porter assays in RAW 264.7 macrophages using the 5′ −243 segment of the murine *IP10* promoter. We cotransfected a dominant-negative mutant of IRF3 (IRF3-DBD) with a deletion of the N-terminal DNA binding domain (133–420) (Lin et al., 1999b) and IκB-DA (pCDNA3-IκBm-ER), a construct that encodes for a fusion protein of the estrogen receptor and an undegradable form of IκB that we have previously shown provides tamoxifen-inducible inhibition of NF-κB (Lee et al., 1999). As shown in Figure 3C, LPS treatment potently induced *IP10* transactivation. However, this effect was inhibited by both IRF3-DBD and IκB-DA.

NF-κB Is Required for Upregulation of Primary Response Genes, While IRF3 Mediates TLR3/TLR4 Specificity

In order to further determine the role of NF-κB in LPS-induced gene expression, we transfected RAW cells with pCDNA3 (mock) or pCDNA3-IκBm-ER (IκB-DA). Single-cell clones stably expressing these constructs were generated by G418 selection and were screened based on inhibition of LPS-induced nitric oxide production (M.E.H. and G.C., unpublished data) and lack of DNA binding activity by EMSA (Figure 4A). Figure 4B shows that LPS stimulation (100 ng/ml) of RAW-mock cells induced rapid upregulation of *IP10*, *IFN*β, and *RANTES*. However, this was almost completely blocked in RAW-IκB-DA cells. These data provide evidence that NF-κB is required for the upregulation of LPS-primary response genes. We then created RAW cell lines stably expressing either full-length IRF3 or IRF3-DBD (Figure 4C). Figure 4D shows Q-PCR analysis of gene expression in wild-type, IRF3, and IRF3-DBD RAW cells treated with LPS (100 ng/ml) (upper panels) or poly I:C (10 μg/ml) (lower panels). IRF3-overexpressing clones had both elevated basal and superinduction of several primary response genes within 1 hr of stimulation, while IRF3-DBD clones had inducible but reduced expression levels as compared to wild-type. Similar results were seen for *ISG15* and *IFIT1* (data not shown). Remarkably, overexpression of IRF3 conferred TLR2 responsiveness to TLR3/TLR4-specific genes (Figure 4E), indicating that IRF3 may be sufficient for the specificity of gene expression observed. To demonstrate that IRF3 was not exerting nonspecific effects, we analyzed *IκBα* gene induction, a direct target of the NF-κB signaling pathway. As shown in Figure 4E (lower right), TLR2 stimulation with PGN induced similar levels of IκBα in RAW-WT and RAW-IRF3 cell lines. The integrity of Q-PCR analyses was controlled by β-*actin* mRNA levels (data not shown). These data support the conclusion that while NF-κB is required for TLR-dependent gene activation, IRF3 is the principal component mediating the TLR3/TLR4 specificity of the primary response genes listed above.

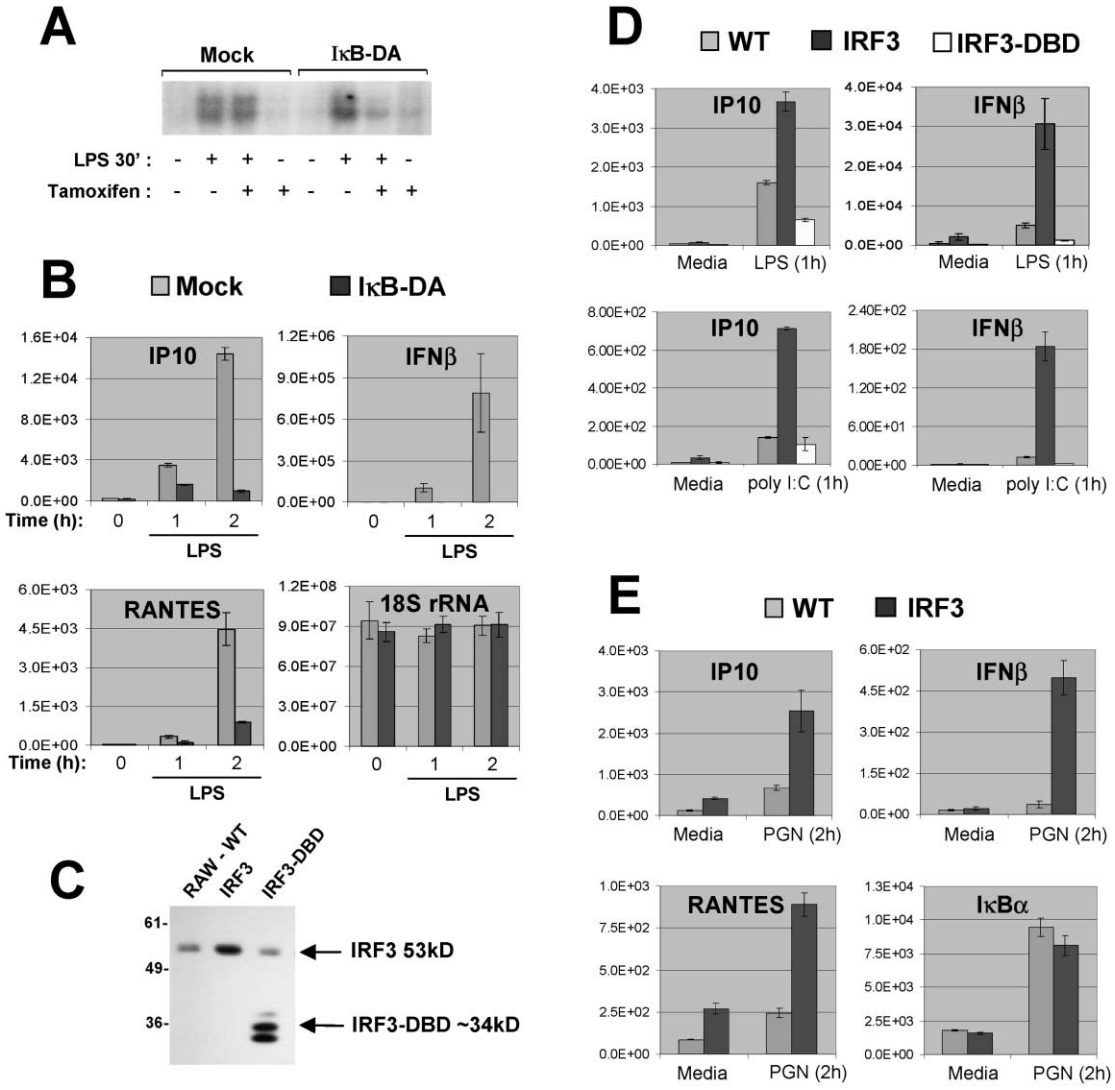


Figure 4. NF-κB Is Required for Activation of Primary Response Genes, While IRF3 Mediates TLR3/TLR4 Specificity

(A) RAW 264.7 clones stably expressing pCDNA3 (mock) or pCDNA3-IκBm-ER (IκB-DA) were treated for 30 min with LPS (100 ng/ml), tamoxifen (200 nM), or both, and NF-κB activity was assayed by EMSA.

(B) RAW-mock and RAW-IκB-DA cell lines were pretreated with tamoxifen (200 nM) for 2 hr and were stimulated with LPS (100 ng/ml) for the indicated time points. RNA was harvested and used for Q-PCR analysis.

(C) Stable expression of pEBB-IRF3 or pEBB-IRF3-DBD in RAW 264.7 cells was detected by Western blotting.

(D) RAW 264.7 macrophages expressing the IRF3 constructs in (C) were stimulated with LPS (100 ng/ml) or poly I:C (10 μg/ml), and RNA was harvested and used for Q-PCR analysis.

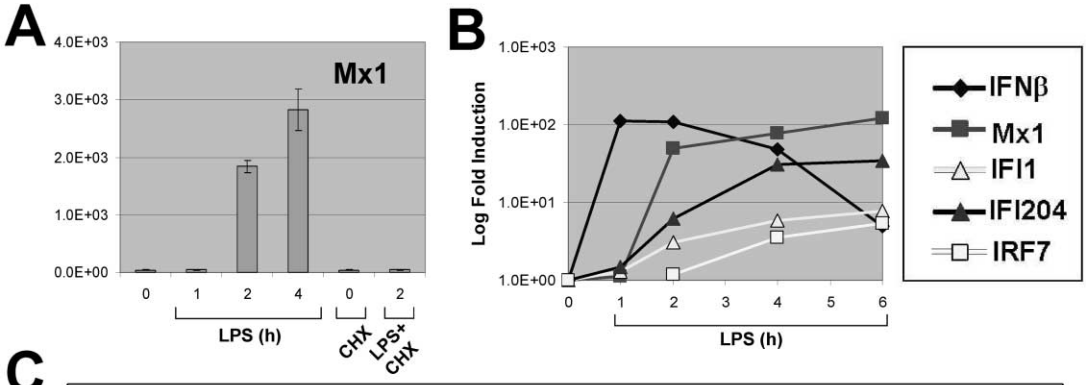
(E) RAW wild-type or IRF3-expressing macrophages were stimulated with PGN (20 μg/ml), and RNA was harvested and used for Q-PCR analysis.

Characterization of TLR3/TLR4-Secondary Response Genes

In the course of our gene expression analysis, we found that several genes initially screened were not induced until 2 hr and were inhibited in the presence of cycloheximide. Figure 5A shows an example of the induction pattern of one secondary response gene, *Mx1*; CHX treatment indicates that prior protein synthesis was required and that this gene is secondarily activated by a LPS-induced protein. Similar results were seen for *IFI1*, *IFI204*, and *IRF7*, and the overall kinetics of activation of these genes versus *IFN*β (primary response) are shown in Figure 5B. *IFN*β is highly upregulated at 1–2 hr, while secondary response genes are induced from 2–6 hr. We focused on four genes—*Mx1*, *IFI1*, *IFI204*, and *IRF7*—whose gene products are thought to be involved in the development of innate immune responses (Figure 5C) (*Mx1* [Arnheiter et al., 1990]; *IFI1* [Collazo et al., 2001]; *IFI204* [Gariglio et al., 1998; Johnstone and Trapani, 1999]; *IRF7* [Sato et al., 2000; Taniguchi et al., 2001]).

TLR3/TLR4-Specific Production of IFNβ Activates Secondary Response Genes Involved in Host Defense

Figure 6A demonstrates that TLR3- or TLR4-agonists but not TLR2- or TLR9-agonists could induce upregula-



C

TLR3/TLR4 Secondary Response Genes*

GENE	Functional Characteristics	Induced by		Confers Resistance to ϕ:	
		LPS	Virus	Bacteria	Virus
Mx1	Cytoplasmic and nuclear GTPase. Resistance to virus thought to be due to indirect modification of cellular functions needed along the viral replication pathway.	+	+	N/D	+
IFI1	GTPase with unknown cellular function.	+	+	+	N/D
IFI204	Nucleolar member of HIN200 Family. Shown to inhibit growth and delay cell cycle progression; possibly by binding to Rb and UBF1.	+	+	N/D	N/D
IRF7	Member of Interferon Regulatory Factor Family. IRF3 and IRF7 shown to be required for positive feedback upregulation of IFN-α/β upon viral infection.	+	+	N/D	N/D

Figure 5. Characterization of TLR3/TLR4 Secondary Response Genes

BMMs were stimulated with LPS (100 ng/ml) for the indicated time points, and RNA was harvested and then analyzed by Q-PCR.
(A) Mx1 gene induction expressed in relative expression units.
(B) Kinetics of activation of *IFN*β versus secondary response genes expressed as fold change (note: log scale).
(C) Summary of TLR3/TLR4 secondary response genes (see text for details). *, secondary-reponse defined as upregulated by LPS (100 ng/ml) at 4 hr but blocked in the presence of cycloheximide (20 μg/ml). ϕ, as demonstrated by studies in transgenic mice. N/D, not determined; HIN, Hematopoietic Interferon-inducible Nuclear Protein.

tion of the secondary response genes. We also found that activation of TLR4 but not TLR2 or TLR9 induced STAT1 phosphorylation (Figure 6B) and that this effect could be blocked by treatment with cycloheximide (data not shown). Type I IFNs (α/β) are known to induce STAT1α/β phosphorylation (Fu, 1992; Schindler et al., 1992), and while *IFN*β is clearly a primary response gene (Figure 2C), we found that no significant upregulation of *IFN*α subspecies mRNA occurred until 4 hr as detected by Q-PCR analysis (data not shown).

In order to investigate whether IFNβ was responsible for the activation of our subset of secondary response genes, we conducted experiments using the cell-free conditioned media (CM) of BMMs treated with TLR-agonists for 2 hr. As shown in Figure 6C, treatment of fresh BMMs with LPS 2 hr CM resulted in rapid activation of the LPS-secondary response genes within 30 min, as opposed to 2 hr of treatment with LPS alone (Figure 5B). The addition of anti-IFNα/β blocking antibodies but not nonspecific rabbit IgG abolished this gene induction, demonstrating that IFNβ in the CM was responsible for this effect. Similar results were seen for *IRF7* (data not shown). Notably, LPS 2 hr CM but not LPS alone could also induce the rapid phosphorylation of STAT1 in 30 min, and this effect could be blocked by addition of anti-IFNβ (Figure 6D). These data together demonstrate that the TLR4-specific upregulation of IFNβ can activate STAT1 and is responsible for the secondary upregulation of *Mx1*, *IFI1*, *IFI204*, and *IRF7*.

TLR3- and TLR4-Activation Inhibits MHV68 Replication

As some of the secondary response genes activated by TLR3 and TLR4 are known to play a role in viral resistance, we next sought to determine if these TLR ligands could directly inhibit the replication of murine γherpesvirus 68 (MHV68). BMMs were simultaneously infected with MHV68 (moi = 5) and treated with various TLR ligands (10, 1, or 0.1 ng/ml lipid A; 100 nM CpG; 10 μg/ml PGN; or 1, 0.1, or 0.01 μg/ml poly I:C) for 48 hr, and replication of viral proteins was then assayed by Western blot analysis. Figure 6E demonstrates that either lipid A (lanes 4–6) or poly I:C (lanes 8–10) treatment could significantly inhibit MHV68 replication in a concen-

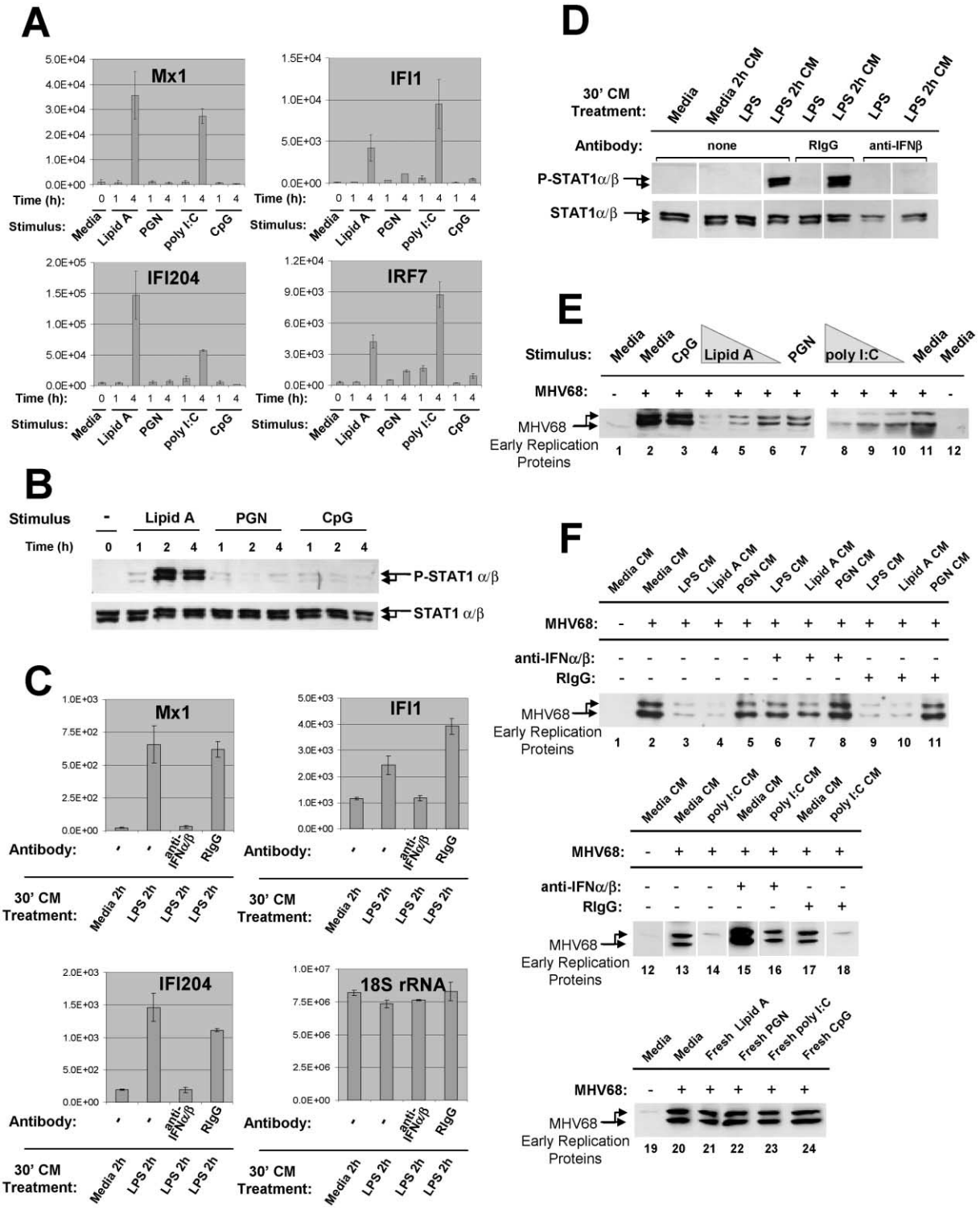


Figure 6. TLR3/TLR4 Stimulation Induces Production of IFNβ and Activates Antiviral Responses

(A) BMMs were stimulated for the indicated time points with the following TLR-agonists: lipid A (1 ng/ml), PGN (20 μg/ml), poly I:C (1 μg/ml), or CpG (100 nM), and RNA was harvested and used for Q-PCR analysis.

(B) BMMs were treated for indicated time points with the following TLR-agonists: lipid A (1 ng/ml), PGN (50 μg/ml), or CpG (100 nM), and 20 μg of protein extract was analyzed by SDS-PAGE immunoblotting using antibody specific for phosphorylated-STAT1 (Y701) or total STAT1.

(C) BMMs were treated for 30 min with cell-free conditioned media (CM) from BMMs treated for 2 hr with media or 100 ng/ml LPS in the presence or absence of 20 μg/ml blocking antibodies or nonspecific rabbit IgG (RIgG) as indicated. RNA was harvested and used for Q-PCR analysis.

(D) STAT1 activation is blocked by anti-IFNβ. BMMs were treated for 30 min with CM as described in (C), and STAT1 phosphorylation was then assayed as in (B).

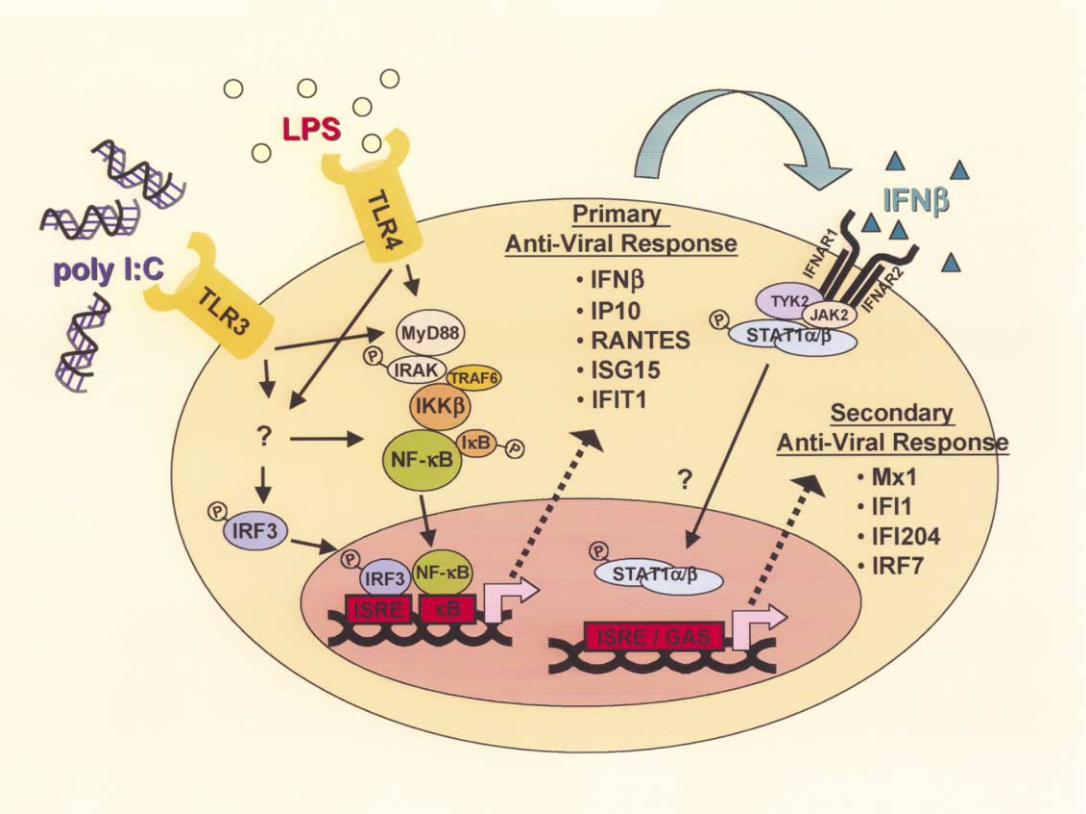


Figure 7. Model of TLR3/TLR4-Specific Antiviral Gene Program

Activation of TLR3 and TLR4 by poly I:C and LPS, respectively, induces the nuclear translocation of IRF3 and NF-κB, which leads to the upregulation of a set of primary response genes. IFNβ is one important cytokine that is produced, activates STAT1, and induces expression of genes that can inhibit viral replication in uninfected cells.

tration-dependent manner, while PGN had a smaller effect (lane 7), and CpG (lane 3) treatment was similar to the media control. During infections performed in the continuous presence of PGN, we repeatedly observed a minor inhibition in MHV68 replication. This was true whether BMMs were treated with 10 or 20 μg/ml PGN, and the inhibition was always considerably weaker than that caused by either 1 ng/ml lipid A or 1 μg/ml poly I:C. These data indicate that among the TLRs tested, TLR3 and TLR4 are the strongest activators of genes that play a role in resistance to viral infection.

We have shown that TLR3 and TLR4 can specifically induce *IFN*β and multiple downstream IFNβ response genes. However, the functional relevance of this signal and subsequent gene program were still undetermined. We therefore designed experiments in which we pretreated NIH3T3 cells (which are hyporesponsive to PAMP treatment) with the cell-free conditioned media (CM) from BMMs stimulated with PAMPs for 3 hr. We then assayed viral replication following 24 hr infection MHV68. Figure 6F shows that while media-treated control samples had significant amounts of viral protein (lane 2), only cells treated with CM from BMMs stimulated with TLR3 or TLR4 ligands were able to suppress viral replication (lanes 3, 4, and 14). Neither PGN CM nor direct treatment with PAMPs had a significant effect (lanes 5 and 21–24). Finally, inhibition of viral replication by TLR3 and TLR4 ligands was specifically abolished by addition of neutralizing antibodies to Type I IFNα/β (lanes 6, 7, and 16). These data indicate that TLR3/TLR4-induced IFNβ mediates a functionally significant role in the innate immune response to viral infection.

Discussion

In this report, we have identified a specific subset of genes induced by stimulation of TLR3/TLR4 and demonstrated that IRF3 and NF-κB are key transcription factors responsible for this gene expression. While NF-κB was commonly activated by several TLRs, IRF3 was shown

(E) BMMs were infected with MHV68 (moi = 5) and simultaneously treated with the indicated TLR ligands (100 nM CpG; 10, 1, or 0.1 ng/ml lipid A; 10 μg/ml PGN; or 1, 0.1, or 0.01 μg/ml poly I:C). After 48 hr, cells were harvested and analyzed for MHV68 replication proteins by immunoblotting using rabbit anti-MHV68 antibodies.

(F) NIH 3T3 cells were pretreated for 3 hr with conditioned media from BMM treated with LPS (100 ng/ml), lipid A (1 ng/ml), PGN (10 μg/ml), or poly I:C (1 μg/ml) in the presence or absence of anti-IFNα/β or nonspecific rabbit IgG (20 μg/ml). Cells were then infected with MHV68 (moi = 1) for 24 hr. MHV68 replication was assayed as described in (E). All results are representative of at least three separate experiments.

to direct the specific induction of a set of primary and secondary genes involved in host defense. Activation of the TLR3/TLR4 signaling pathway was also found to potently inhibit viral infection by MHV68 through the autocrine/paracrine production of IFNβ. Overall, we have described the signaling network that leads to the automatic and sequential activation of specific genes in response to dsRNA or LPS/Lipid A—a TLR3/TLR4-specific antiviral gene program (Figure 7). These data suggest that TLR3 and TLR4 have evolutionarily diverged from other members of the TLR family and can trigger important antiviral responses through activation of IRF3.

Initially, our microarray data indicated that in B cells, LPS and CD40L activate many similar sets of genes for overlapping biological functions, such as cell survival, proliferation, metabolism, and immunological isotype switching (data not shown). CD40L specifically upregulated a subset of genes involved in cell adhesion, migration, and germinal center formation (Dadgostar et al., 2002), while LPS induced inflammatory cytokines (such as *TNF*α, *IL-1*β, and *IL-6*) and a subset of "interferon-associated" genes, as well as other poorly characterized genes with no previously described roles in TLR4 signaling (see Supplemental Data at http://www.immunity.com/cgi/content/full/17/3/251/DC1). Our data further confirm results observed in other published LPS-gene expression studies. However, it is notable that the LPS-specific genes listed in Figure 1A show remarkable overlap with genes upregulated by viral infection as indicated by viral gene expression studies (Geiss et al., 2001; Li et al., 2001; Suzuki et al., 2000; Zhu et al., 1998). We and others have also confirmed that some LPS-primary response genes, such as *IP10* and *RANTES*, are also secondarily upregulated by autocrine production of IFNβ (data not shown) (Ohmori and Hamilton, 2001).

The specific activation of IRF3 by TLR3 and TLR4 led us to investigate gene expression with extensive titration of TLR-agonists. We found that increasing doses of PGN (25–50 μg/ml) could induce mild upregulation of Type I interferon through an IRF3-independent mechanism, particularly at later time points. However, TLR3/TLR4-agonists at small doses (1 ng/ml LPS/lipid A or 1 μg/ml poly I:C) caused more than a 50-fold increase in gene expression by 2 hr (data not shown). In addition, TLR2- and TLR9-agonists were unable to induce detectable IRF3 nuclear translocation at any concentration tested. While the nuances of regulation of each individual gene are unique and outside the scope of this paper, the contribution of IRF3 to the enhanceosomes of some of these genes has been well documented in models of viral infection (Lin et al., 1999a; Wathelet et al., 1999). Activation of IRF3 after viral infection has been shown to be the first step in activation of a "gene program" that includes a positive feedback loop of Type I IFNs and IRF family members (Taniguchi et al., 2001). Interestingly, while the data presented here indicate that TLR3 and TLR4 activate gene expression by a similar mechanism at early time points, several lines of evidence suggest that even these receptors diverge with respect to their activation of innate antiviral responses. Specifically, TLR3 induced a stronger activation of IRF3 (Figure 3A), and this correlated with higher levels of *IFN*β (Figure 2C). Gene expression profiles from longer stimulations showed that while TLR4 induces *IFN*β expression from 1 to 4 hr, TLR3 induces much higher levels of *IFN*β with extended kinetics, with maximal levels at 8 hr (S.A.V. and G.C., unpublished data). This suggests that the TLR family of receptors have evolved to exert a stimulus-specific modulation of antiviral responses while retaining pathways common to all TLRs that lead to production of proinflammatory genes such as *TNF*α (Figure 2C).

While TLR4 can recognize some viral components, a critical question still remains—how and why do bacterial products such as LPS activate this pathway? The role of IRF3 or IFNβ in bacterial infection is not well understood. However, some recent reports have highlighted the ability of IFNβ to synergistically induce important components of the antimicrobial response such as iNOS and IFNβ (Jacobs and Ignarro, 2001; Yaegashi et al., 1995). On the other hand, another report found that Type I interferons are associated with increased susceptibility to bacterial infection by *Mycobacterium tuberculosis* (Manca et al., 2001). Clearly, more work must be done to determine the true functional outcomes of TLR3/TLR4 activation.

While much is known about the biochemical events downstream in the TLR signaling pathways, evidence of increasing complexity between the individual receptors has led to a renewed interest in the biological role of the TLRs. Few studies have been able to conclusively prove increased susceptibility to a natural pathogen in TLR-deficient mice. However, the amazing detection capacities and evolutionary conservation of the TLRs strongly argue for an important functional role. Currently, it is unclear exactly how TLRs bind their ligands or cooperate with each other. It is possible that TLR3 and TLR4 may cooperate in the detection and response to certain viruses and may act separately or in conjunction with yet other TLRs to recognize other pathogens. Further work is certainly required to clarify this question.

Undoubtedly, activation of either TLR3 or TLR4 involves a much larger and more complex gene program than illustrated in this report. However, our findings show that these receptors can specifically activate signaling pathways that render cells more resistant to viral infection. TLR ligands can exert both immunostimulatory and toxic effects in vivo, and the data presented here identify distinct signaling pathways that lead to inflammatory or antiviral responses. The identification of a specific gene program that enhances innate antiviral activity may provide new directions for therapeutic treatments of viral infections. In addition, the development of pharmacological drugs that would allow manipulation of such a gene program might allow us to enhance the innate immunity in conditions where the adaptive immune system is compromised.

Experimental Procedures

Microarray and Clustering Analysis

B cell isolation, target preparation, and hybridization using Affymetrix Mu6500 microarrays were performed as described previously (Dadgostar et al., 2002). Differential expression data was analyzed by Affymetrix Microarray Suite 4.0 software. Average difference change values were then normalized, and the genes were clustered by the uncentered correlation average linkage hierarchical clustering algorithm using Cluster. Data were then visualized as a dendogram using Treeview software (http://rana.lbl.gov/EisenSoftware.htm).

Cell Culture and Reagents

Murine BMMs were differentiated from marrow from 6- to 10-week-old C57B/6 mice as previously described (Chin et al., 2002). BMMs were maintained in 1× DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin, and 30% L929 conditioned medium, and purity was assayed to be 94%–99% $CD11b^+$. The RAW 264.7 murine macrophage cell line (ATCC: TIB-71) was maintained in 1× DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. In order to study TLR activation, we used the following: 055-B5 *E. coli* LPS (Sigma), F-583 *E. coli* lipid A (Sigma), *S. aureus* peptidoglycan (Sigma), CpG oligonucleotides (Invitrogen), poly I:C (Pharmacia), and cycloheximide (Sigma). The dosage of poly I:C used was lowered from 10 μg/ml to 1 μg/ml for long-term experiments to ensure viability of treated cells.

RNA Quantification

RNA was isolated for both Northern blotting and quantitative real-time PCR (Q-PCR) using a standard guanidium isothiocyanate method. Northern blotting was done as previously described (Lee et al., 1999) and was hybridized using a *RANTES* cDNA fragment (IMAGE Clone: 832342, Research Genetics). For Q-PCR, RNA was quantitated and 2 μg of RNA was reversed transcribed using Superscript II (Invitrogen) according to the manufacturer's instructions with either oligo-dT or random hexamer as primers. Q-PCR analysis was done using the iCycler thermocycler (Bio-Rad). Q-PCR was conducted in a final volume of 25 μL containing: Taq polymerase, 1× Taq buffer (Stratagene), 125 μM dNTP, SYBR Green I (Molecular Probes), and Fluoroscein (Bio-Rad), using oligo-dT cDNA or random hexamer cDNA as the PCR template. Amplification conditions were: 95°C (3 min), 40 cycles of 95°C (20 s), 55°C (30 s), 72°C (20 s). The following primers were used to amplify a specific 100–120 bp fragment of the following genes: *RANTES* 5′, GCCCACGTCAAGGAG TATTTCTA; *RANTES* 3′, ACACACTTGGCGGTTCCTTC; *Mx1* 5′, AAA CCTGATCCGACTTCACTTCC; *Mx1* 3′, TGATCGTCTTCAAGGTTTC CTTGT; *IFI1* 5′, CCAGAGCATGGGAAAGAGGTT; *IFI1* 3′, CCGGACCT CTGATAGGACACTG; *IFI204* 5′, TTGGCTGCAATGGGTTCAT; *IFI204* 3′, AGT GGGATATTCATTGGTTCGC; *IRF7* 5′, ACAGGGCGTTTTATC TTGCG; *IRF7* 3′, TCCAAGCTCCCGGCTAAGT; *IP10* 5′, CCTGCCCA CGTGTTGAGAT; *IP10* 3′, TGATGGTCTTAGATTCCGGATTC; *ISG15* 5′, CAGGACGGTCTTACCCTTTCC; *ISG15* 3′, AGGCTCGCTGCAGT TCTGTAC; *IFIT1* 5′, GGCAGGAACAATGTGCAAGAA; *IFIT1* 3′, CTCA AATGTGGGCCTCAGTT; *18S* 5′, CCGCGGTTCTATTTTGTTGGT; *18S* 3′, CTCTAGCGGCGCAATACGA; *IFN*β 5′, AGCTCCAAGAAAGGACG AACAT; *IFN*β 3′, GCCCTGTAGGTGAGGTTGATCT; *IκBα* 5′, CTGCAG GCCACCAACTACAA; *IκBα* 3′, CAGCACCCAAAGTCACCAAGT; β-*Actin* 5′, AGGTGTGCACCTTTTATTGGTCTCAA; and β-*Actin* 3′, TGTATGAAGGTTTGGTCTCCCT.

Plasmid Constructions

The full-length and dominant-negative IRF3-expression plasmids were created by PCR amplifiction of IRF3 cDNA (IMAGE clone: 3666172) using either IRF3(1-420) 5′-CAGGACTGATCAACCATGGA AACCCCGAAACCGCGGATT-3′ or IRF3-DBD(133-420) 5′-CAGGAC ATCCATGCACTCCCAGGAAAACCTACCGAAG-3′ in conjunction with the 3′ primer 5′-CAGGACGCGGCCGCGATATTCCAGTGGCCTGGA AGTC-3′. Fragments were cloned into the BglII/Not1 or BamHI/NotI sites of pEBB-puro. pCDNA3-IκBm-ER was constructed as described (Lee et al., 1999). The −243 *IP10* pCAT plasmid was a kind gift of Thomas A. Hamilton.

Transfections and CAT Assays

All transfections were done using Superfect (Qiagen) according to manufacturer's instructions. All plasmids were purified using Endo-free Maxiprep (Qiagen). Single-cell clones for IκB and IRF3 constructs were selected for using 1 mg/ml G418 and 2.5 μg/ml puromycin, respectively. Chloramphenicol acetyl-transferase (CAT) assays were done as described elsewhere (Ohmori and Hamilton, 1993).

Immunoblotting, EMSA, and In Vitro Kinase Reactions

Cell fractionation and nuclear Western immunoblotting were done as described elsewhere (Lee et al., 1999). Anti-IRF3 was obtained from Zymed, anti-USF-2, and anti-STAT1 from Santa Cruz Biotechnologies, and antibodies specific to the phosphorylated forms of STAT1 and c-Jun were obtained from Cell Signaling Technologies. IFNβ blocking experiments employed an anti-IFNβ antibody (R&D Systems), anti-Type I IFNα/β (Access Biomedical, Inc.), or nonspecific Rabbit-IgG (Sigma) at final concentrations of 20 μg/ml. Cells were lysed in modified RIPA buffer, extracts were quantitated using either the Bradford assay reagent (Bio-Rad) or the BCA Protein Quantitation kit (Pierce), and 20 μg of protein was loaded in each lane and separated by SDS-PAGE. Gels were transferred to nitrocellulose filters and immunoblotted using the antibody manufacturers' recommended instructions. For detection of MHV68, rabbit anti-MHV68 was used as described by Wu et al. (2001). To detect activation of JNK following TLR activation, in vitro kinase reactions were performed as previously described (Dadgostar and Cheng, 2000). EMSA was done as previously described (Lee et al., 1999).

Virus Production, Infection, and Harvesting

Murine γherpesvirus 68 (MHV68) was produced and titered as previously described (Wu et al., 2001). For infection of macrophages, cells were simultaneously treated with PAMPs and infected with MHV68 at an moi of 5. Following an incubation period of 48 hr, cells were lysed in Laemmli buffer and 10% of total volume was subjected to SDS-PAGE, transferred to nitrocellulose, and MHV68 proteins detected by Western blotting.

For NIH3T3 experiments, macrophages were first treated with PAMPs in the presence or absence of anti-Type I IFNα/β (Access Biomedical, Inc.), or nonspecific rabbit-IgG (Sigma) for a period of 3 hr. Conditioned medias were then collected and used to treat NIH3T3 cells for another 3 hr. Cells were then infected with MHV68 at an moi of 1. Following an incubation period of 24 hr, cells were harvested and processed for viral content as described above.

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