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Molecular Determinants of Crosstalk between Nuclear Receptors and Toll-like Receptors

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Summary

Nuclear receptors (NRs) repress transcriptional responses to diverse signaling pathways as an essential aspect of their biological activities, but mechanisms determining the specificity and functional consequences of transrepression remain poorly understood. Here, we report signal- and gene-specific repression of transcriptional responses initiated by engagement of toll-like receptors (TLR) 3, 4, and 9 in macrophages. The glucocorticoid receptor (GR) represses a large set of functionally related inflammatory response genes by disrupting p65/interferon regulatory factor (IRF) complexes required for TLR4- or TLR9-dependent, but not TLR3-dependent, transcriptional activation. This mechanism requires signaling through MyD88 and enables the GR to differentially regulate pathogen-specific programs of gene expression. PPARγ and LXRs repress overlapping transcriptional targets by p65/IRF3-independent mechanisms and cooperate with the GR to synergistically transrepress distinct subsets of TLR-responsive genes. These findings reveal combinatorial control of homeostasis and immune responses by nuclear receptors and suggest new approaches for treatment of inflammatory diseases.

Introduction

Members of the nuclear receptor superfamily play diverse roles in the regulation of development, homeostasis, and immune responses by both positively and negatively regulating gene expression. The glucocorticoid receptor (GR) is prototypic of a subset of ligand-dependent nuclear receptors that integrate host immune responses with physiological circuits that are required for maintenance of necessary organ functions. The ability of GR to repress transcriptional responses to inflammatory signals is an essential component of its homeostatic functions and a primary mechanism by which natural and synthetic GR agonists exert anti-inflammatory effects in a variety of disease settings (Reichardt et al., 2001, and reviewed in De Bosscher et al. [2003]). Negative regulation of inflammatory responses is thought to result, at least in part, from the ability of GR to interfere, by transrepression, with the activities of other signal-dependent transcription factors that include NF-κB and activator protein-1 (AP-1) family members (Jonat et al., 1990; Ray and Prefontaine, 1994; Schüle et al., 1990). Numerous models have been proposed for GR-mediated transrepression, including direct interactions with NF-κB components (Caldenhoven et al., 1995; Liden et al., 1997; Scheinman et al., 1995b), regulation of components of signal-transduction pathways involved in NF-κB and AP-1 activation (Auphan et al., 1995; Caelles et al., 1997; Scheinman et al., 1995a), competition for essential coactivators (Kamei et al., 1996; Sheppard et al., 1998), alternative utilization of coactivators (Kassel et al., 2004; Rogatsky et al., 2001; Scheinman et al., 1995b), recruitment of corepressors (Nissen and Yamamoto, 2000), and modifications of core transcription factors (De Bosscher et al., 2000; Nissen and Yamamoto, 2000). However, most of these models have been developed based on analysis of limited sets of specific target genes and general applicability to broad programs of gene expression activated during inflammatory responses have not been established.

Anti-inflammatory activities have also been documented in vivo and/or in vitro for several other members of the nuclear-receptor family, including estrogen receptors (ERs) (McKay and Cidlowski, 1999), vitamin D receptors (VDRs) (Nagpal et al., 2001), peroxisome proliferator-activated receptors (PPARs) (Devchand et al., 1996; Jiang et al., 1998; Ricote et al., 1998), and LXRs (Castrillo and Tontonoz, 2004). PPARs and LXRs are regulated by fatty acid and cholesterol metabolites, respectively, and were initially characterized as nuclear receptors that play critical roles in lipid homeostasis (Issemann and Green, 1990; Janowski et al., 1996). Emerging evidence suggests that their ability to counterregulate inflammatory responses plays important roles in both immunity and metabolic control (Castrillo and Tontonoz, 2004; Joseph et al., 2004; Valledor et al., 2004).

Here, we have used toll-like receptor (TLR) signaling as a model system to explore mechanisms by which different members of the nuclear-receptor superfamily repress proinflammatory programs of gene expression. GR, PPARγ, and LXR agonists were found to repress both common and distinct subsets of TLR target genes through the use of nuclear-receptor- and TLR-specific transrepression mechanisms. Combinations of ago-

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nists for GR, PPARγ, and LXRs resulted in additive or synergistic inhibition of a subset of TLR4-target genes both in cultured macrophages and in vivo, consistent with the simultaneous targeting of these genes by distinct mechanisms. These findings suggest that nuclear receptors function in a combinatorial manner to coordinately regulate the evolution of host immune responses.

Results

Nuclear Receptors Inhibit Overlapping but Distinct Subsets of LPS-Inducible Genes

Gene-expression profiling experiments were initially performed to identify LPS-inducible genes in primary macrophages that were sensitive to transrepression by the GR agonist dexamethasone (Dex; Figure 1A). The observation that about half of the LPS-inducible genes were Dex-sensitive raised the questions of how GR discriminated between sensitive and resistant genes and whether these two classes of genes exert distinct biological functions. Recent findings indicate that NCoR corepressor complexes occupy a subset of NF-κB and AP-1 target genes under basal conditions and are cleared in a signal-dependent manner as a prerequisite to transcriptional activation (Ogawa et al., 2004; Perissi et al., 2004). Because some NCoR target genes were also subject to Dex-mediated repression, microarray experiments were performed using *NCoR*$^{-/-}$ macrophages to determine whether NCoR was required for Dex sensitivity (Ogawa et al., 2004). Lack of NCoR had no impact on GR-mediated repression (Figure 1B), indicating the utilization of NCoR-independent mechanisms. To investigate whether different nuclear receptors repress a common set of LPS target genes, gene expression profiling experiments were performed using nuclear-receptor-specific agonists for PPARγ (rosiglitazone [Ro] and GW7845), LXRα/β (GW3965 and T1317), VDR (1,25-$(OH)_2$vitamin D_3), ER (17β-estradiol; E_2), and RARs (all-*trans* retinoic acid; atRA). These experiments demonstrated that each agonist exerted an overlapping but distinct impact on LPS-dependent gene expression (Figures 1C and 1D). GR, LXR, and PPARγ agonists were the most potent inhibitors of the LPS response, with VDR-, ER-, and RAR-specific agonists exerting relatively modest inhibitory effects under these conditions (Figure 1C). Sensitivity to repressive effects of GR, PPARγ, or LXR agonists did not correlate with degree of responsiveness to LPS (Figure 1C), absolute expression levels, or a requirement for the p65 component of NF-κB (data not shown and see Figure S1 in the Supplemental Data available with this article online).

p65/IRF3 Complexes Mediate Signal-Specific Inhibition of Transcriptional Responses

Computational motif discovery methods were used to search for potential transcriptional regulatory elements mediating LPS-dependent activation and nuclear-receptor-mediated transrepression. The sequence cAAAct GAAAg was identified as the most highly significant motif enriched in the promoter sequences of LPS target genes (Figure 2A). This motif is nearly identical to consensus IRF3 and interferon (IFN)-sensitive response element (ISRE) sequences recognized by IRF3 and the type I IFN-inducible ISGF3 complex. Binding of poly I:C to TLR3 and LPS to TLR4, respectively, activates IRF3 and induces ISRE-mediated gene activation (Pitha, 2004; Servant et al., 2002). We therefore performed expression-profiling experiments to compare the impact of GR, PPARγ, and LXR agonists on transcriptional responses of macrophages to TLR3 and TLR activation (Figure 2C). TLR4 signals through both Myd88-dependent and TRIF-dependent pathways, while TLR3 exclusively signals through the TRIF-dependent pathway (Akira and Takeda, 2004; Pitha, 2004). Despite this difference, the qualitative and quantitative pattern of genes induced more than 3-fold by LPS and poly I:C at the 6 hr time point were very similar (Figure 2B). In parallel, gene expression profiling experiments were performed to identify IRF3-target genes by comparing LPS responses of wild-type and *IRF3*$^{-/-}$ macrophages (Sato et al., 2000; Figure 2C; Table S1).

As in the case of LPS-dependent gene expression, GR, PPARγ, and LXR agonists repressed both common and nuclear-receptor-specific targets of poly I:C-inducible genes (Figure 2C). However, despite the overall similarity in the sets of genes that were transcriptionally activated by LPS and poly I:C, the patterns of GR-, PPARγ-, and LXR-mediated transrepression were significantly different, indicating that nuclear-receptor transrepression is regulated in a signal-specific manner. In particular, a substantial number of genes that were Dex sensitive when activated by LPS became Dex-resistant when activated by poly I:C, illustrated for *IP10* and *Ifit1* in Figure 2D.

Unexpectedly, nearly all of the highly inducible LPS- and poly I:C target genes that were Dex sensitive when activated by LPS but Dex resistant when activated by poly I:C were also highly dependent on IRF3 for LPS induction (Figure 3A and data not shown). The promoters for many of the genes exhibiting this pattern of expression contained proximal IRF3/ISRE sequences, exemplified by *Ifit1* (Figure 3B). In contrast, while PPARγ and LXR agonists also inhibited a significant number of IRF3-dependent genes, the signal-specific pattern of sensitivity and resistance differed (Figures 2C and 3A). This pattern therefore suggested a mechanistic link between IRF3 and signal-specific transrepression by GR. To test this hypothesis, we evaluated the ability of GR to repress transcriptional activation of an artificial promoter constructed to exclusively contain ISRE elements, which was activated by LPS and poly I:C (Figure 3C). Significantly, the induction of the ISRE-dependent reporter was strongly inhibited by Dex when LPS was used as a stimulus, but not when poly I:C was used as a stimulus (Figure 3C).

While TLR3 and TLR4 signaling both lead to activation of IRF3, recent studies suggest that p65/RelA functions as an essential coactivator of IRF3 in the case of TLR4 signaling, but not in case of TLR3 signaling (Wietek et al., 2003). The p65 requirement for LPS induction of an ISRE-dependent gene was confirmed by experiments demonstrating markedly impaired activation of the *Ifit1* gene in NF-κB-deficient macrophages (Figure 3D). These observations raised the possibility that GR inhibited IRF3-target genes in response to TLR4 signaling, but not TLR3 signaling, by targeting the p65 require-

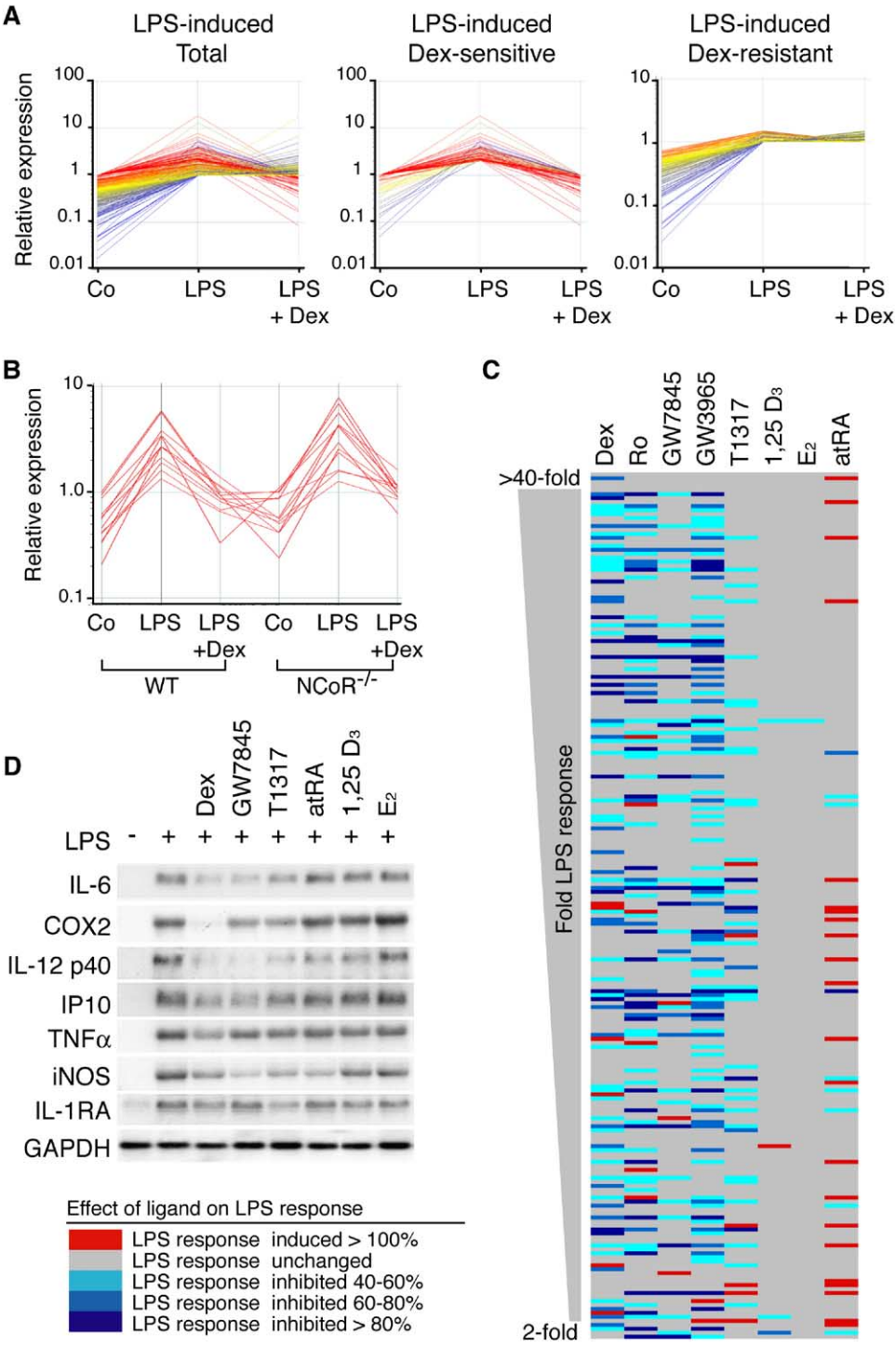

Figure 1. Inhibition of LPS-Dependent Gene Expression by Nuclear-Receptor Agonists

(A) Relative expression of LPS-inducible genes in peritoneal macrophages under control conditions and after 6 hr of LPS treatment in the absence or presence of 1 μM Dex. Left panel, genes induced by LPS >2-fold. Middle panel, genes induced by LPS >2-fold and inhibited by Dex more than 50% (Dex-sensitive). Right panel, genes induced by LPS >2-fold and resistant to Dex.

(B) Dex-mediated transrepression of LPS-inducible genes in macrophages derived from fetal liver-derived macrophages of wild-type (WT) and *NCoR*$^{-/-}$ mice. The illustrated gene expression values are for the 12 most highly repressed genes in wild-type macrophages.

(C) Effect of nuclear-receptor agonists on responsiveness of 208 LPS target genes in peritoneal macrophages. Genes are ordered based on magnitude of average LPS induction over 4 experiments from >40-fold at the top to 2-fold at the bottom. Genes in which the LPS response was not altered by agonist treatment are illustrated in gray. Red indicates ligand-dependent upregulation and blue ligand-dependent downregulation of the LPS response. The magnitude of the effect is indicated by the key at lower left.

(D) Confirmation of negative regulation of LPS target genes by Northern blotting. Macrophages were treated with LPS for 6 hr in the presence of 1 μM concentrations of the indicated agonists.

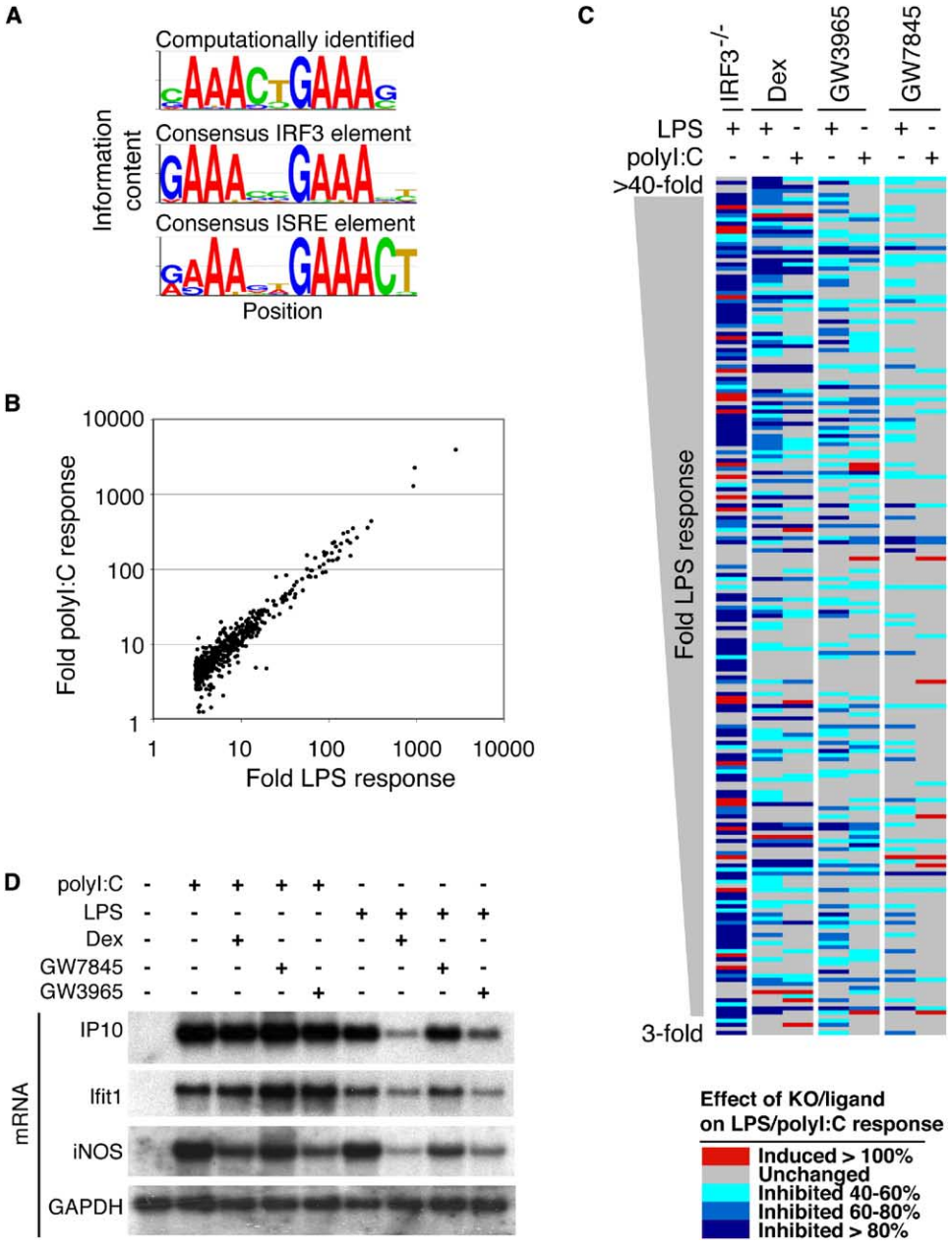

Figure 2. Differential Repression of TLR Responses by GR, PPARγ, and LXRs

(A) Identification of IRF3 binding sites as highly enriched sequence motifs in the promoters of LPS-inducible genes. The top sequence logo is representative of the most significant motif present in the promoters of genes that are LPS-inducible and not present in promoters of non-LPS-inducible genes.

(B) A scatter plot illustrating fold responses to poly I:C compared to genes activated at least 3-fold by LPS.

(C) Effect of IRF3 deficiency and GR (Dex), LXR (GW3965), or PPARγ (GW7845) agonists on transcriptional responses to LPS and poly I:C. The panel illustrates the 208 most highly induced LPS-responsive genes. Sensitivity of each gene to loss of IRF3 (column 1) or treatment with Dex, GW3965, and GW7845 is color coded as indicated in the key at the bottom.

(D) Confirmation of signal-specific repression of LPS- and poly I:C-inducible genes by GR-, LXR-, and PPARγ-specific agonists. Macrophages were treated with LPS or poly I:C for 6 hr in the presence of 1 μM concentrations of the indicated agonists.

ment. We therefore characterized the composition of activation complexes bound to the proximal promoter region of *Ifit1* in primary macrophages by chromatin immunoprecipitation (ChIP) assay. These experiments demonstrated that IRF3 and CBP were prominently recruited to the ISRE-containing promoter in response to both LPS and poly I:C (Figure 3E). In contrast, p65 was specifically recruited to the ISRE in response to LPS, but not in response to poly I:C. Interestingly, the recruitment of p65 to the ISRE in response to LPS was largely inhibited by Dex, but not by PPARγ or LXR agonists (Figure 3E). Similar findings were obtained using ChIP to evalu-

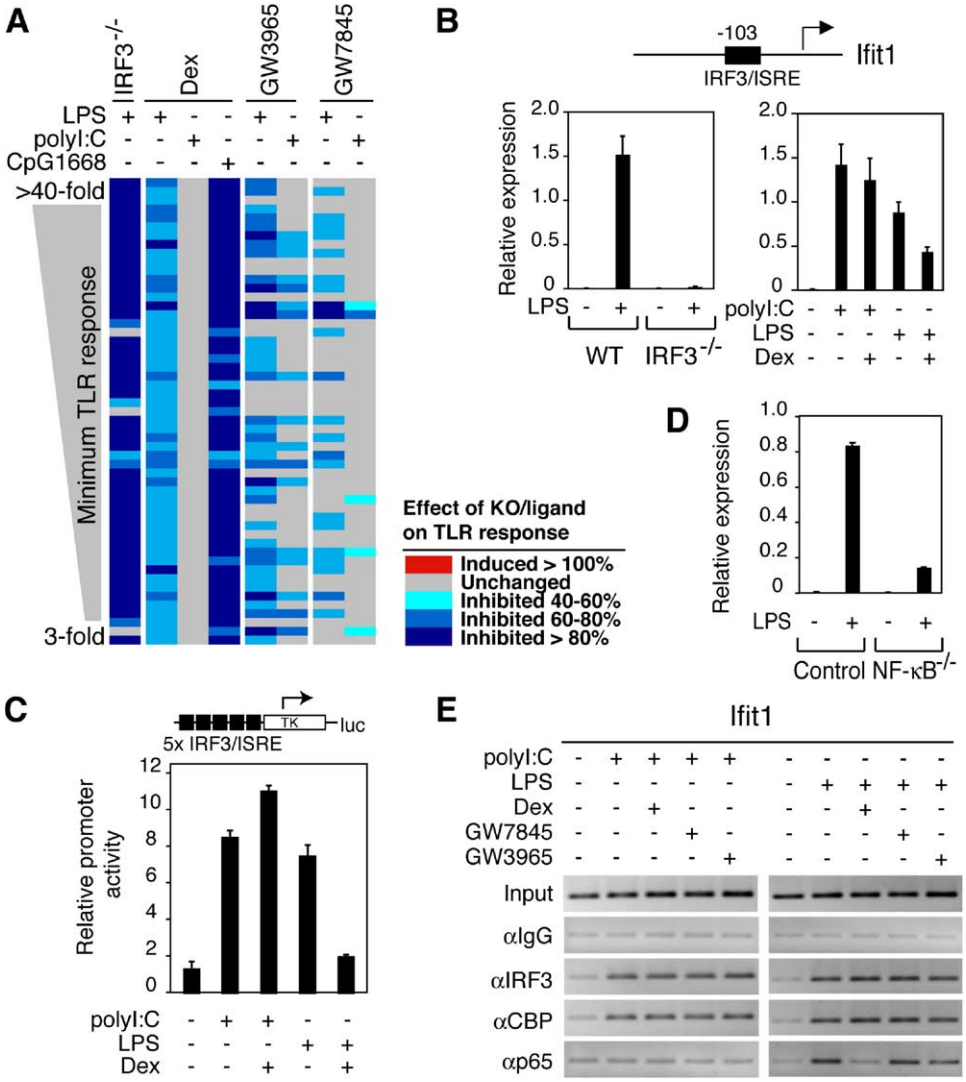

Figure 3. Signal-Specific Repression by GR Correlates with a Requirement for IRF3 for Transcriptional Activation

(A) Transcriptional responses of 54 genes highly induced by LPS, poly I:C, and CpG1668, exhibiting sensitivity to Dex when activated by LPS (column 2) and resistance to Dex when activated by poly I:C (column 3). The dependence of the LPS response on IRF3 is indicated in the first column. Effects of IRF3 deficiency or nuclear-receptor agonists are color coded as in Figure 2C.

(B) Promoter structure and expression profile of *Ifit1* in response to LPS, poly I:C, and Dex in wild-type (WT) and *IRF3*$^{-/-}$ peritoneal macrophages. Error bars represent standard deviations.

(C) An ISRE-specific promoter exhibits LPS-specific repression by Dex. U373 cells were transfected with a 5× ISRE-Luc reporter plasmid. Cells were treated with the indicated combinations of LPS (100 ng/ml), poly I:C (50 μg/ml), and Dex and analyzed for luciferase activity 18 hr later. Error bars represent standard deviations.

(D) Expression of *Ifit1* in response to LPS (1 μg/ml) in control and NF-κB$^{-/-}$ fetal liver-derived macrophages.

(E) p65 recruitment to the proximal promoter region of *Ifit1* is specifically induced by LPS and inhibited by activation of GR. Primary macrophages were treated with LPS (100 ng/ml), poly I:C (50 μg/ml), and the indicated agonists for GR, PPARγ and LXRs for 1 hr. ChIP assays were performed with antibodies against IRF3, CBP, p65 and control IgG, respectively. Immunoprecipitated DNA was analyzed by PCR using primers specific for the promoter.

ate the composition of activation complexes bound to the artificial promoter construct exclusively containing ISRE elements (Figure S2).

Previous studies have suggested that nuclear-receptor interactions with p65 are involved in transrepression (Liden et al., 1997; Scheinman et al., 1995b), but how this interaction could account for receptor-, signal-, and gene-specific repression has not been established. We therefore evaluated the possibility that GR inhibited LPS induction of the ISRE promoter through direct interactions with p65. In vitro interaction assays confirmed that the GR-DBD strongly interacted with both full-length p65 and further narrowed this interaction to the N-terminal Rel-homology domain (RHD; Figure 4A). In contrast, the PPARγ-DBD and the LXRα-DBD exhibited minimal interaction with p65 in vitro (Figure 4A). These results were confirmed by mammalian two-hybrid assays using a Gal4DBD-p65 fusion protein as

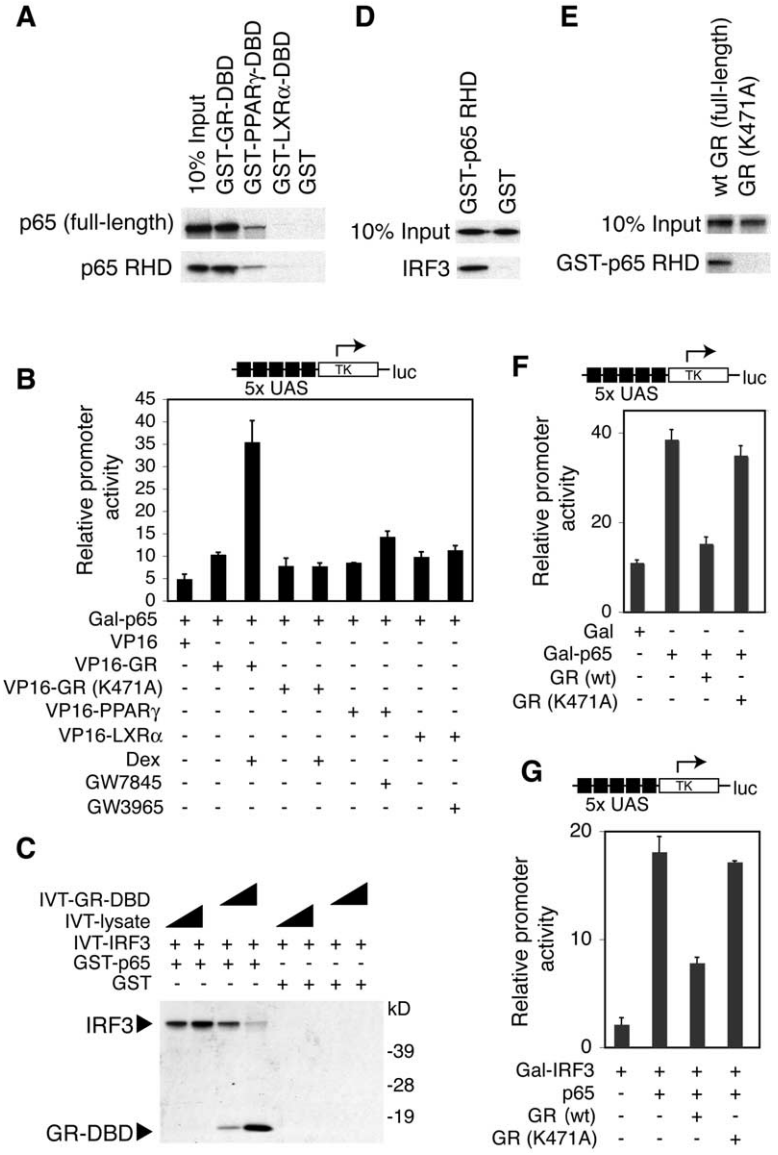

Figure 4. GR Specifically Inhibits the Interaction of p65 with IRF3

(A) GR-DBD preferentially interacts with the p65 RHD. GST pull-down assays were performed using the indicated GST-NR-DBD fusion proteins and in vitro translated full-length p65 or p65 RHD, respectively.

(B) Wild-type GR preferentially interacts with p65 in vivo in a ligand-dependent manner. The mammalian two-hybrid assay was performed in RAW264.7 cells using Gal-p65 as bait and the indicated VP16 nuclear-receptor fusion proteins as preys in the presence and absence of agonists. Error bars represent standard deviations.

(C) GR-DBD inhibits interaction of IRF3 with p65. GST pull-down assays were performed using GST-p65 and increasing amounts of in vitro translated full-length IRF3 and/or GR-DBD as indicated.

(D) IRF3 interacts with the p65 RHD in vitro. GST pull-down assays were performed using GST-p65 RHD and in vitro translated full-length IRF3.

(E) GR-DBD mutant GR^{K471A} is unable to interact with the p65 RHD in vitro. GST pull-down assays were performed using GST-p65 RHD and in vitro translated full-length GR and GR^{K477A}, respectively.

(F) Inhibition of Gal-p65 transactivation by liganded wild-type GR but not by GR^{K477A}. Endogenous GR expression in mouse RAW264.7 cells was knocked down by pretreatment with a GR-specific siRNA for 48 hr. Cells were then transfected with expression vectors for Gal4 (Gal), Gal4-p65 (Gal-p65), wild-type human GR or GR^{K477A} as indicated in the presence of Dex. Error bars represent standard deviations.

(G) Coactivation of Gal-IRF3 by p65 is inhibited by liganded wild-type GR but not by GR^{K477A}. Endogenous GR expression in RAW264.7 cells was knocked down by pretreatment with a GR-specific siRNA for 48 hr prior to transfection with expression vectors for human wild-type GR or GR^{K477A} and treatment with Dex as indicated. Error bars represent standard deviations.

bait and VP16 fusions with full-length GR, PPARγ, or LXRα as preys (Figure 4B). In vitro binding assays demonstrated that a glutathione-S-transferase (GST)-p65 fusion protein also interacted with IRF3 through the RHD (Figures 4C and 4D). Addition of increasing amounts of GR-DBD to the binding reaction led to decreased IRF3 interaction with p65 and a concomitant increase in the binding of the GR-DBD (Figure 4C). These findings suggest that GR and IRF3 compete for the same binding site and that GR preferentially interacts with p65.

To determine whether the interaction of GR with p65 was relevant to its repression function, we evaluated a GR mutant in which lysine 471 in the second zinc finger of the DBD was changed to alanine (GR^{K471A}) based on a previous report that a corresponding mutant is defective for inhibition of p65 activity (Liden et al., 1997). In contrast to wild-type GR, GR^{K471A} exhibited little interaction with p65 in vitro or in vivo in mammalian two-hybrid assays (Figures 4B and 4E), and GR^{K471A} lacked inhibitory activity against Gal-p65-mediated transactivation (Figure 4F). Consistent with these in vitro findings, wild-type GR was able to inhibit p65-dependent transactivation of a Gal-IRF3 fusion gene, while GR^{K471A} was not (Figure 4G), suggesting that direct interaction of GR and p65 is required for GR-mediated transrepression of IRF3. Taken together, these findings support a model in which the requirement of ISRE-containing genes for p65 as a coactivator following LPS activation, but not poly I:C stimulation, accounts for the LPS-specific sensitivity of these genes to transrepression by GR.

IRF3/p65 Complexes Mediate Gene-Specific Inhibition of Transcriptional Responses

In addition to the IRF3 binding motif, κB elements were also highly enriched in promoter regions of LPS-inducible genes (Table S1). However, the presence of these

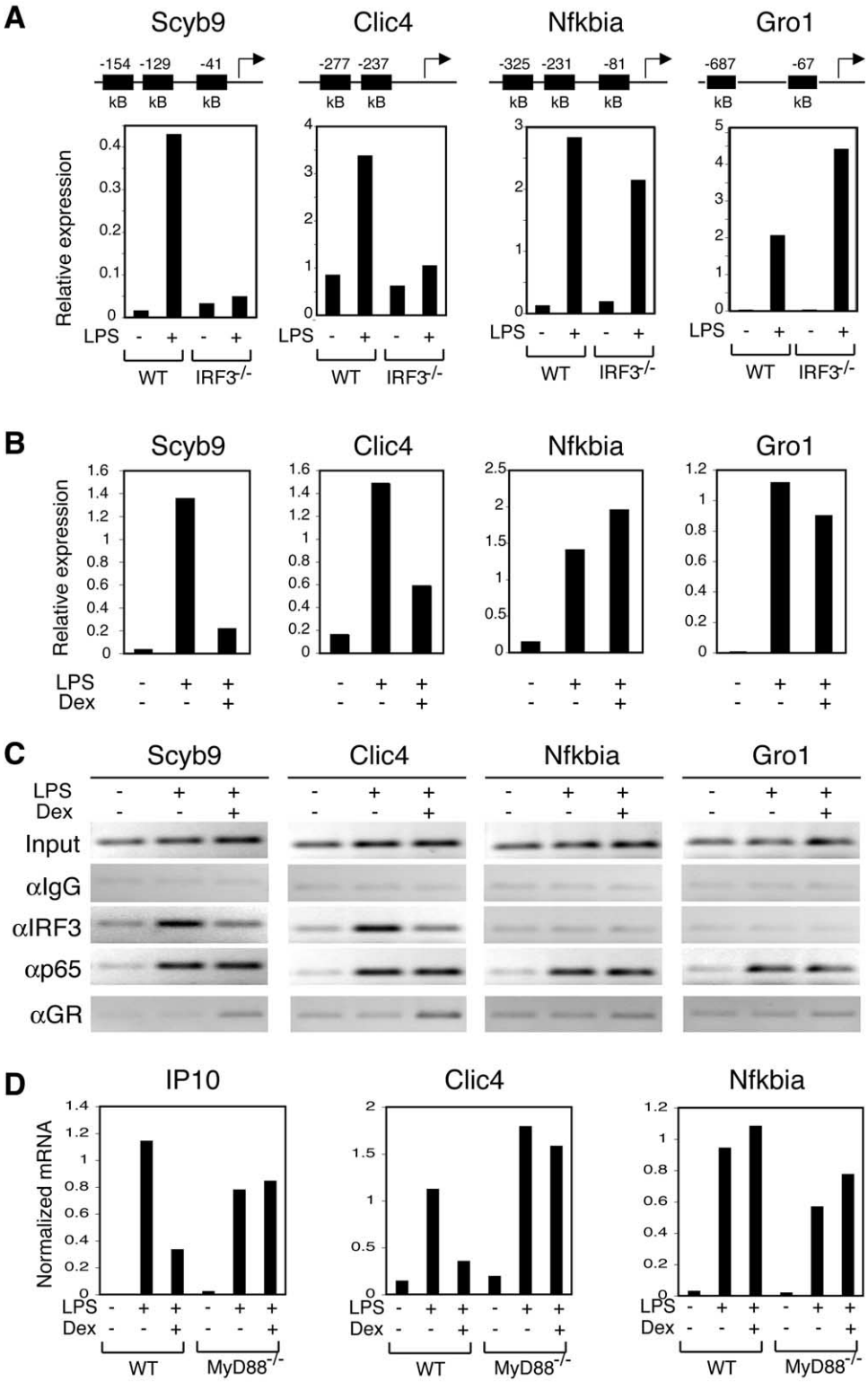

Figure 5. Utilization of IRF3 as a Coactivator of p65 Determines Gene-Specific Sensitivity

(A) Location of NF-κB sites in proximal promoter regions of Scyb9, Clic4, Nfkbia, and Gro1, and their expression profiles in response to LPS in wild-type (WT) and *IRF3*$^{-/-}$ peritoneal macrophages. Error bars represent standard deviations.

(B) Expression profiles of Scyb9, Clic4, Nfkbia, and Gro1 in response to LPS and Dex in wild-type peritoneal macrophages. Error bars represent standard deviations.

(C) Recruitment of IRF3 to the proximal promoter regions of Scyb9 and Clic4, but not Nfkbia or Gro1, in response to LPS. Recruitment of IRF3 was largely inhibited by Dex. Proximal regions of Scyb9, Clic4, Nfkbia, and Gro1 promoters that includes an NF-κB site were analyzed by ChIP assays using the indicated antibodies. Crosslinking was performed 1 hr after treatment with LPS and Dex.

(D) Expression profiles of IP10, Clic4, and Nfkbia in response to LPS and Dex in control (WT) and *MyD88*$^{-/-}$ peritoneal macrophages. Gene expression was determined by real-time quantitative PCR. Error bars represent standard deviations.

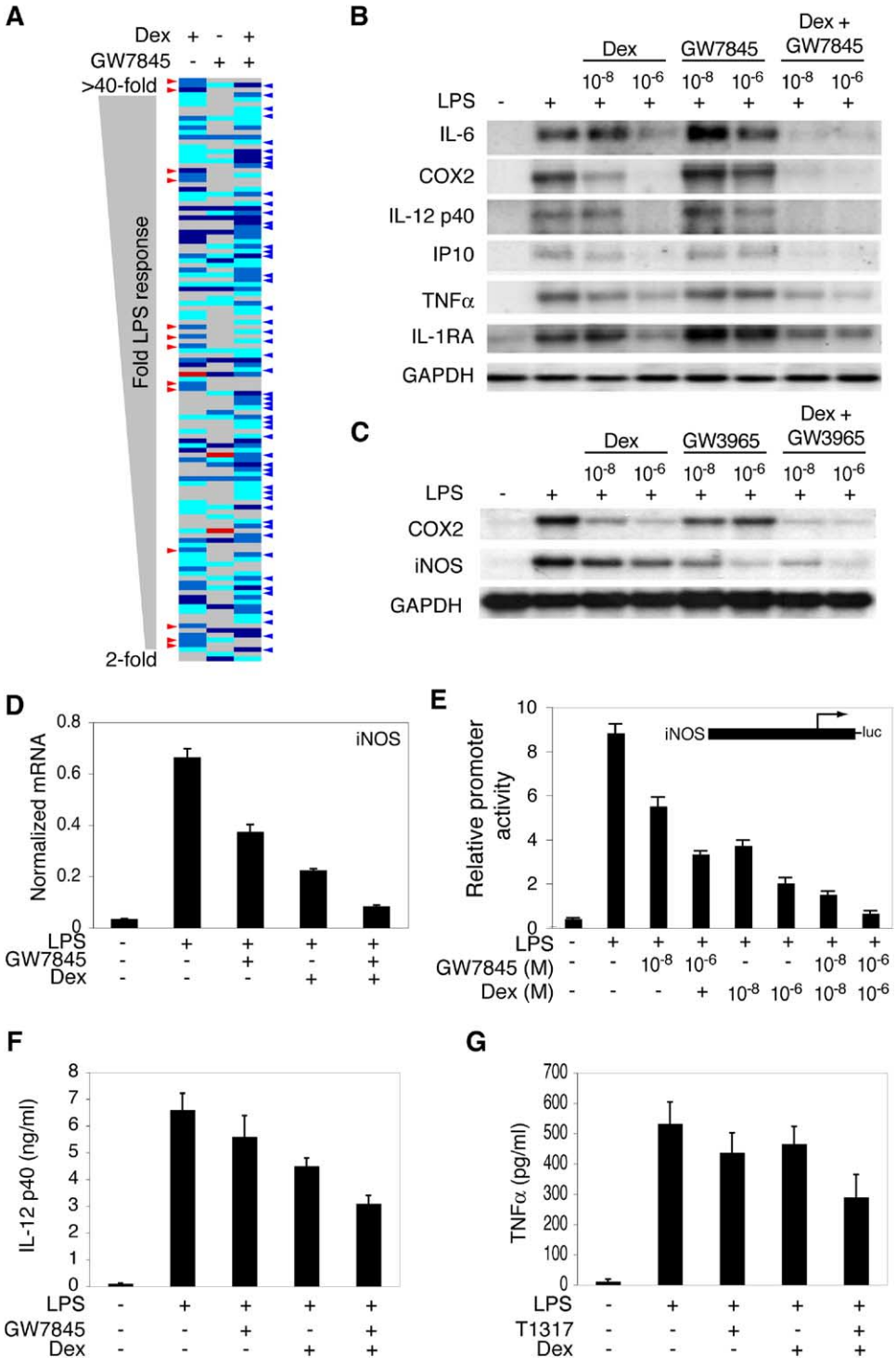

Figure 6. GR and PPARγ Function in a Combinatorial Manner to Inhibit LPS Responses

(A) Combinatorial interactions between GR and PPARγ agonists at a genome-wide level. Peritoneal macrophages were stimulated with LPS in the absence or presence of Dex alone, GW7845 alone, or the combination of Dex plus GW7845. The panel illustrates LPS-target genes exhibiting a >40% reduction of the LPS response in the presence of at least one agonist. Effects of agonists on the LPS response are color coded according to the legend in Figure 1C. Red arrows indicate genes in which one agonist abolished strong inhibitory effects of the other agonist. Blue arrows indicate genes in which the combination of Dex and GW7845 resulted in stronger inhibition of the LPS response than either agonist alone.

(B) Confirmation of combinatorial effect of GR and PPARγ agonists on regulation of LPS-target genes by Northern blotting. Macrophages were treated with LPS for 6 hr in the presence of the indicated concentrations (10 nM, 1 μM) of agonists.

(C) Confirmation of combinatorial effect of GR and LXR agonists on regulation of LPS-target genes by Northern blotting.

(D) Confirmation of combinatorial effect of GR and PPARγ agonists (1 μM) on iNOS expression by real-time quantitative PCR. Error bars represent standard deviations.

sequences did not correlate with signal- or gene-specific patterns of regulation. NF-κB binding sites were identified in promoters of genes that were sensitive to nuclear-receptor agonists following activation by LPS or poly I:C (e.g., *iNOS*, Figure 2D), in promoters of genes that were nuclear-receptor-sensitive following LPS but not poly I:C activation (e.g., *Clic4*, Figure 5A and data not shown), and in promoters of genes that were nuclear receptor resistant regardless of the signal (e.g., *Nfkbia* and *Gro1*, Figure 5A and data not shown). In each case, transcriptional responses to LPS required p65 (Figure S1). The recent finding that a subset of NF-κB sites appear to determine the utilization of IRF3 as a coactivator of p65 (Leung et al., 2004) suggested the possibility that this might be a basis for gene-specific sensitivity to repression by GR. To examine this, we chose the *Scyb9* and *Clic4* genes because they were highly IRF3 dependent, Dex sensitive NF-κB target genes that did not contain ISRE motifs within their proximal promoter or distal upstream regions (Figures 5A and 5B). The *Nfkbia* and *Gro1* genes were chosen for comparison because they were highly induced by LPS in an IRF3-independent manner, were Dex resistant, contained well-characterized κB elements, and lacked proximal or distal ISRE elements (Figures 5A and 5B). ChIP experiments revealed that p65 was recruited to each of these genes in response to LPS, as expected (Figure 5C). IRF3 was recruited to the proximal promoter regions of *Scyb9* and *Clic4* in response to LPS, but not to the *Nfkbia* or *Gro1* promoters, consistent with the requirement of *Scyb9* and *Clic4* for IRF3 for activation and confirming a gene-specific recruitment of IRF3 to a subset of p65 target genes. Significantly, treatment with Dex had no effect on the recruitment of p65 to any of these four target genes but inhibited the recruitment of IRF3 to the *Scyb9* and *Clic4* promoters, coincident with ligand-dependent recruitment of GR to these promoters (Figure 5C).

MyD88 Dictates GR Sensitivity of IRF3/7-Dependent Gene Expression

Because TLR3 and TLR4 activate IRF3 and NF-κB through the TRIF-dependent pathway, while TLR4, in addition, activates NF-κB and MAP kinases via the MyD88-dependent pathway, these observations raised the possibility that glucocorticoid sensitivity was dictated by the utilization of the MyD88-dependent pathway. To initially test this hypothesis, we determined the profile of dexamethasone-sensitive genes in macrophages treated with immunostimulatory DNA (CpG1668) to activate TLR9, which exclusively couples to the MyD88-dependent pathway (Hacker et al., 2000; Krug et al., 2004). As in the case of polyI:C-stimulated cells, the overall profile of transcriptional activation induced by CpG1668 was very similar to that induced by LPS. Remarkably, 100% of the genes that were highly induced by all three TLR agonists and were Dex resistant when activated by polyI:C but Dex sensitive when activated by LPS, were also Dex sensitive when activated by CpG1668 (Figure 3A). Furthermore, the quantitative extent of Dex-mediated repression was more pronounced following TLR9 stimulation than TLR4 stimulation in nearly every case (Figure 3A). To determine whether Dex sensitivity of the response to LPS requires signaling through the MyD88 pathway, experiments were performed in *MyD88*$^{-/-}$ macrophages (Adachi et al., 1998). In these cells, LPS activation is entirely TRIF dependent (Akira and Takeda, 2004; Kawai et al., 2001). Remarkably, IRF3-dependent genes that were Dex sensitive in wild-type cells became Dex resistant in *MyD88*$^{-/-}$ cells, regardless of whether they contained ISRE or κB elements (Figure 5D). These findings suggest that signaling through the MyD88-dependent pathway specifies Dex sensitivity of this set of genes.

GR, PPARγ, and LXR Function in a Combinatorial Manner to Inhibit LPS Responses

The observation that GR, PPARγ, and LXR agonists repressed overlapping but distinct sets of LPS target genes by p65-dependent and p65-independent mechanisms raised the possibility that they might exert combinatorial effects on inflammatory responses. To test this hypothesis, gene expression profiling experiments were performed to characterize LPS responses in the presence or absence of combinations of saturating concentrations of GR, PPARγ, and LXR agonists. The results of this analysis for the combination of Dex and the PPARγ agonist GW7845 are illustrated in Figure 6A, restricted to the subset of genes transrepressed by at least one agonist. While several examples were observed in which nuclear-receptor-specific inhibitory effects of one agonist were reversed by addition of the second agonist (red arrows), the major impact of the combination of agonists was to increase the strength of inhibition of a subset of LPS target genes (blue arrows). These results were confirmed by additional experiments that examined the concentration dependence of combinatorial interactions by Northern blot analysis and quantitative PCR analysis of representative target genes (Figures 6B and 6D). Low concentrations of Dex and GW7845 (10 nM) that exerted relatively little repressive effects when used individually could act synergistically in combination (Figure 6B). Parallel studies of combinations of Dex and GW3965 also demonstrated

(E) Combinatorial interactions between GR and PPARγ at the promoter levels. RAW 264.7 cells were transfected with a luciferase reporter plasmid under transcriptional control of the iNOS promoter, PPARγ, and RXRα expression plasmids. Cells were treated with the indicated combinations of LPS, Dex, and GW7845 and analyzed for luciferase activity 24 hr later. Error bars represent standard deviations.

(F) In vivo effects of combinations of GR and PPARγ agonists on the response to intraperitoneal injection of LPS. Six C57BL6 mice were pretreated with the indicated combinations of GW7845 (1 mg/kg) and Dex (1 mg/kg) for 7 days, injected intraperitoneally with LPS (1 mg) and circulating levels of IL-12 p40 were measured by ELISA 8 hr later. Error bars represent standard deviations.

(G) In vivo effects of combinations of GR and LXR agonists on the response to intraperitoneal injection of LPS. Six C57BL6 mice were pretreated with the indicated combinations of Dex (1 mg/kg) and T1317 (10 mg/kg) for 7 days, injected intraperitoneally with LPS (1 mg) and circulating levels of TNFα were measured by ELISA 6 hr later. Error bars represent standard deviations.

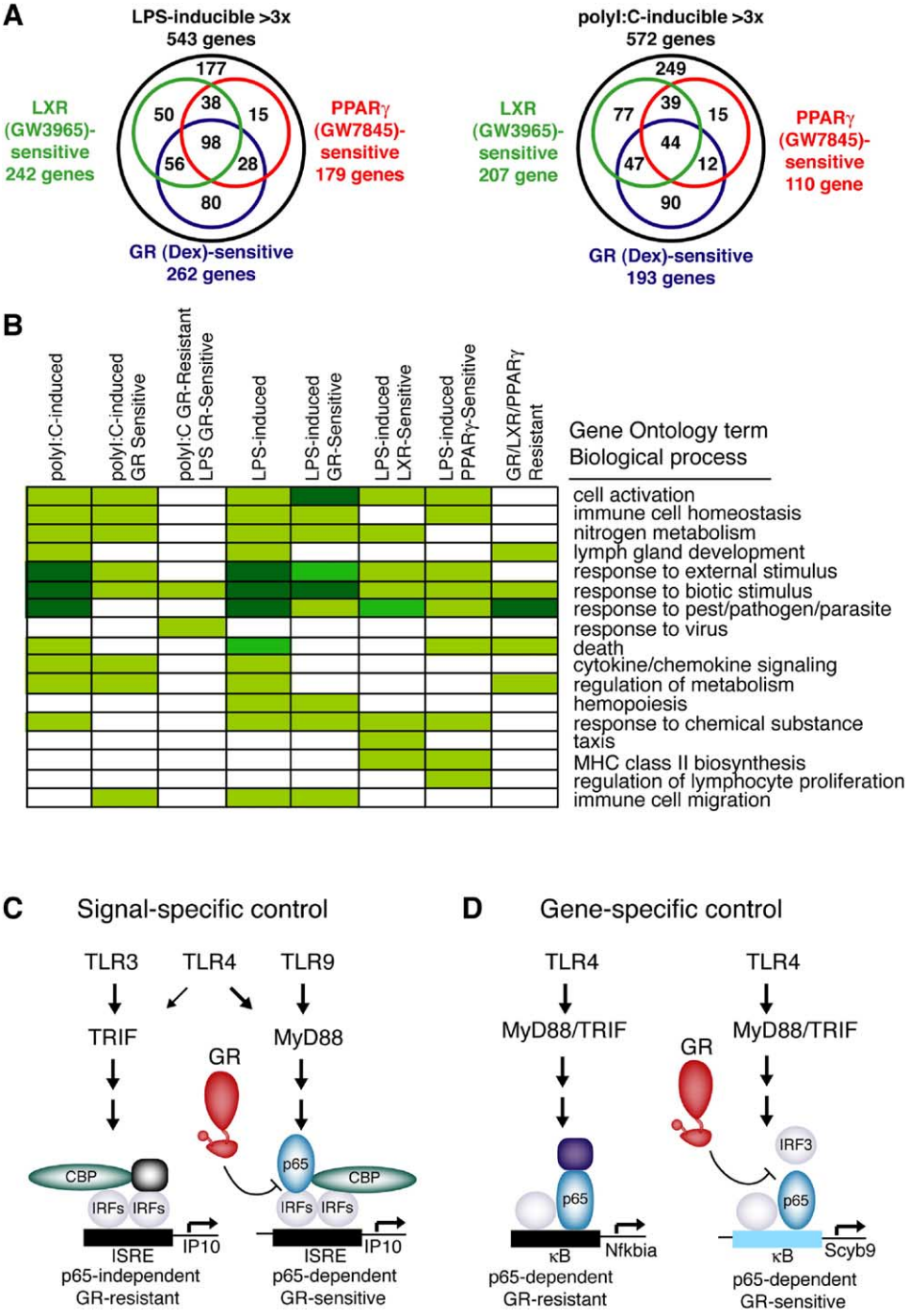

Figure 7. Receptor-, Signal-, and Gene-Specific Counterregulation of Inflammatory Responses by GR, PPARγ, and LXRs

(A) Venn diagrams derived from the complete data set used to generate Figures 2B and 2C indicating sensitivity of LPS- and poly I:C-responsive genes to GR, PPARγ, and LXR-specific agonists.

(B) Representative functional annotations corresponding to Biological Process terms derived from the Gene Ontology database that were significantly enriched in the sets of LPS- and poly I:C-responsive genes. p represents the probability of obtaining the indicated number n genes within the category by chance determined as previously described (Ogawa et al., 2004). Color-coding corresponds to the following p values; light green, p less than 0.01; medium green, p less than 0.0001; dark green, p less than 10^6.

(C) Model for signal-specific GR-mediated transrepression, determined by utilization of p65 as an obligate TLR4-specific coactivator of IRF3. IRF-mediated activation of ISRE-containing genes by TLR4 and TLR9 through MyD88-pathway requires that p65 function as a signal-specific coactivator. The p65/IRF interaction is disrupted by liganded GR, resulting in transrepression. TLR3-specific activation of IRF3 through the TRIF pathway is p65 independent and hence GR resistant.

(D) Model for gene-specific GR-mediated transrepression, determined by utilization of IRF3 as an obligate promoter-specific coactivator of NF-κB. The IRF3/p65 interaction is disrupted by liganded GR, providing an explanation for promoter-specific inhibition of the LPS response.

additive or synergistic effects on LPS target genes (Figure 6C and data not shown).

To determine whether combinatorial effects of GR, PPARγ, and LXR agonists acted at the promoter level, iNOS promoter activity was evaluated in RAW264.7 cells. The iNOS promoter was chosen for this analysis because the endogenous iNOS gene was subject to combinatorial inhibition by GR, PPARγ, and LXR agonists, its transcriptional activation requires binding sites for NF-κB (Lowenstein et al., 1993), and maximum responses to LPS required IRF3 (data not shown). As shown in Figure 6E, GR and PPARγ agonists inhibited iNOS promoter activity in a dose-dependent manner. When cells were treated with the combination of Dex and GW7845, at least additive effects with respect to inhibition of LPS response were observed at both saturating and nonsaturating concentrations of ligands (Figure 6E). Similar results were observed for the combination of GR and LXR agonists (data not shown).

Although GR-mediated repression did not require NCoR (Figure 1B), we recently found that the ability of PPARγ to repress LPS activation of the iNOS promoter required NCoR (G.P. and C.K.G., unpublished data). These findings indicate that at least two distinct receptor-specific mechanisms are utilized by GR and PPARγ to repress LPS activation of the iNOS promoter, providing a potential explanation for synergistic repression when GR and PPARγ agonists are used in combination.

To investigate whether combinatorial interactions between PPARγ agonists and Dex observed in primary macrophages would also occur in an in vivo model system, we evaluated the IL-12 p40 subunit, as this was synergistically repressed in primary macrophages by GW7845 and Dex, but not by the combination of Dex and T1317. Consistent with these findings, treatment of mice with the combination of GW7845 and Dex prior to injection with LPS resulted in significantly greater inhibition of circulating IL-12 p40 than either agonist alone (Figure 6F). For the combination of GR and LXR agonists, we evaluated TNFα, based on synergistic inhibition by Dex and T1317 in primary macrophages. Treatment of mice with the combination of Dex and T1317 resulted in significantly greater inhibition of circulating tumor necrosis factor α (TNFα) levels following injection with LPS than observed following either agonist alone (Figure 6G).

Biological Functions Associated with Nuclear-Receptor-Sensitive and Nuclear-Receptor-Resistant TLR Target Genes

The identification of distinct subsets of TLR3 and TLR4 target genes exhibiting nuclear-receptor-sensitive or nuclear-receptor-resistant profiles raises the question of whether these genes participate in distinct biological processes. Venn diagrams illustrating the overlapping and distinct subsets of genes subject to transrepression by GR, PPAR, and LXR following macrophage activation by LPS and poly I:C are illustrated in Figure 7A. To investigate the potential meaning of these findings at a biological level, statistical analysis of the functional annotations associated with specific sets of differentially regulated genes was performed using annotations provided by the Gene Ontology (GO) database (Gene Ontology Consortium, 2001). A subset of this analysis is presented in Figure 7B, illustrating major categories including immune cell homeostasis, response to virus, cytokine, chemokine signaling, etc. The results of this analysis suggest significant functional differences in the sets of nuclear-receptor-sensitive and nuclear-receptor-resistant LPS-target genes. For example, the set of genes that was activated by LPS and resistant to GR, PPARγ, and LXR agonists was enriched for functional annotations linked to metabolism (Figure 7B). In addition, transcriptional activation of the core components of the NF-κB pathway by LPS or poly I:C was almost completely resistant to repression by all three nuclear-receptor agonists (Figure S3B). We also placed data for GR-mediated repression of LPS- and poly I:C-inducible genes on KEGG pathway maps (Kanehisa, 1996) and provide an example for the TLR signaling pathway in Figure S4. This figure indicates that components of the TLR signaling pathway in addition to NF-κB factors are Dex resistant, while TLR-activated chemokines and cytokines exhibit differential patterns of sensitivity that relate to proinflammatory effects and chemotaxis.

GR, PPARγ, and LXR regulated functionally overlapping sets of genes, but also targeted genes in functionally related groups in a nuclear-receptor-specific manner. For example, the list of repressed genes with functional annotations linked to hemopoiesis by the Gene Ontology Consortium reached statistical significance for Dex, but not LXR or PPARγ agonists (Figure 7B). Repressive actions of each nuclear-receptor ligand on specific genes involved in immune cell migration, differentiation, and activation are illustrated in Figure S3B. Overall, Dex inhibited a larger number of genes involved in immune cell activation to a greater extent than LXR or PPARγ agonists, which may explain in part why LXR and PPARγ agonists are not as effective as Dex in acute models of inflammation. Significant differences in effects of the three receptor-specific agonists on chemokine gene expression were observed, suggesting that each receptor may play a context-specific role in regulating recruitment of specific immune cells to sites of inflammation.

Discussion

Signal-Specific, Gene-Specific, and Nuclear-Receptor-Specific Transrepression

The present studies have used a combination of gene expression profiling and molecular analysis to investigate nuclear-receptor-specific and combinatorial mechanisms of transrepression by nuclear receptors. These observations extend the spectrum of nuclear-receptor- and promoter-specific inhibition of signal-dependent gene expression, demonstrating that GR, PPARγ, and LXR repress overlapping but distinct subsets of inflammatory response genes, consistent with the large number of mechanisms that have been proposed for negative regulation by nuclear receptors (De Bosscher et al., 2003). The observation that a significant set of genes that were sensitive to nuclear-receptor-dependent repression when activated through TLR4 became resistant to repression when activated through TLR3 also indicates that transrepression programs mediated by

GR, PPARγ, and LXRs are regulated in a signal-specific manner. Of the 262 genes scored as being LPS inducible and Dex sensitive in these studies, at least 85 genes fit with the hypothesis that disruption of IRF3/p65 complexes is a quantitatively important component of the transrepression mechanism. Taken together with the results of studies in *MyD88*$^{-/-}$ macrophages (Figure 5D) and the patterns of gene expression following TLR9 activation (Figure 3A), these findings support a unifying model in which TLR signaling through MyD88 specifies glucocorticoid sensitivity of IRF-dependent genes through the utilization of IRF/p65 complexes (Figure 7C). As IRF7 is involved in TLR9-MyD88-dependent gene activation (Kawai et al., 2004), these results imply that the mechanism of GR-mediated repression operates through both IRF3 and IRF7. These findings thus reveal a mechanism of signal-specific transrepression that is utilized by a large group of functionally interrelated genes. Distinct regions of GR appear to be involved in mediating repression of AP-1 target genes (Bladh et al., 2005) and it will be of interest to explore signal-specific modulation of transrepression in response to other proinflammatory cytokines that induce AP-1 and STAT transcription factors, such as TNFα and IFNβ.

Conversely, the ability of IRF3 to function as an essential coactivator of p65 on a subset of NF-κB target genes provides an explanation for how transrepression by GR can be achieved in a gene-specific manner (Figure 7D). NF-κB target genes that are resistant to GR-mediated transrepression are predicted to utilize other classes of coactivators, such as Bcl3 (Leung et al., 2004), that may prevent the interaction of GR with DNA bound NF-κB. Consistent with this, ChIP experiments demonstrated recruitment of GR to the Dex-sensitive Scyb9 and Clic4 promoters. A significant number of Dex-sensitive NF-κB target genes are not IRF3-dependent, indicating a requirement for additional mechanisms. Virtually all of the Dex-sensitive poly I:C-inducible genes were also Dex sensitive when activated by LPS and CpG1668 (Figure S3A), suggesting the utilization of common, signal-independent transrepression mechanisms for this class of genes that remain to be defined.

Physiological Implications for Cellular Responses to Bacterial and Viral Pathogens

The observation that TLR-responsive genes exhibit different sensitivities to repression by nuclear receptors suggests that they play distinct biological roles in determining cellular responses to infection and other inflammatory processes. By specifically targeting p65/IRF3 complexes, GR is able to discriminate signals initiated by TLRs that either do or do not couple to the MyD88 signaling pathway, providing a biological rationale for the context-specific utilization of these complexes. The prediction arising from these studies that the antiviral program mediated by TLR3 should be resistant to GR-mediated repression while the antiviral program elicited by activation of TLR9 should be GR sensitive is consistent with clinical experience. Innate immune responses to herpes simplex virus infections involve TLR9 (Krug et al., 2004; Lund et al., 2003), and steroid use is generally contraindicated in the setting of HSV infections because this may exacerbate the severity of infection.

In addition to defining combinatorial control of inflammatory responses at the promoter level, these studies also suggest a higher level of regulation that serves to integrate both local and systemic signaling pathways. In the case of GR, the endogenous corticosteroid ligands are produced in the adrenal cortex under the control of the hypothalamic-pituitary-adrenal axis. Circulating corticosteroids diffuse into cells and bind to GR in virtually all tissues with subnanomolar affinities, providing coordinate regulation of gene expression at the whole-body level. In contrast, PPARγ and LXRs are activated by metabolites of fatty acids and cholesterol, respectively, that are produced locally within the cell and bind with relatively low affinity. In addition, the expression of PPARγ and LXRα and the production of regulatory ligands are determined by local cytokines and other regulatory systems (Ricote et al., 2004). Taken together, these observations suggest a model in which GR and PPARγ/LXR integrate systemic and local regulatory signals so as to coordinate transcriptional responses to infection throughout the body.

Clinical Implications

Nuclear receptors are important targets of drugs used in a variety of human disease settings. In many cases, the ability to achieve desirable therapeutic effects with a natural or synthetic nuclear-receptor agonist is limited by undesirable or unacceptable side effects. For example, glucocorticoids are potent anti-inflammatory drugs but can cause or exacerbate hypertension, diabetes, obesity, and dyslipidemia. Emerging information on the ability of selective modulators of nuclear receptors to alter the specificity of coactivator and corepressor recruitment raises new possibilities for the development of novel pharmaceutical agents (Smith and O'Malley, 2004). The present studies suggest an alternative and potentially complementary strategy to leverage desirable therapeutic effects while minimizing side effects. Using chronic, steroid-dependent inflammatory diseases as an example, it is possible that anti-inflammatory actions of synthetic glucocorticoids could be achieved at lower doses with fewer side effects by simultaneous administration of PPARγ or LXR agonists. Further investigation of these combinatorial mechanisms may provide new insights into how nuclear receptors control signal-activated transcription and lead to novel strategies for treatment of inflammatory diseases.

Experimental Procedures

Cell Culture

Thioglycollate-elicited macrophages were isolated by peritoneal lavage 3 days following peritoneal injection of 2.5 ml 3% thioglycollate (DIFCO). Cells were plated in RPMI medium 1640 and 10% fetal bovine serum and washed; after 5 hr the medium was removed and cells were fed with fresh medium containing 0.5% fetal bovine serum. LPS (Sigma) was used at a concentration of 100 ng/ml. Nuclear-receptor ligands were used at 1 μM concentrations except as indicated. Fetal liver-derived macrophages generated from E14.5 embryo liver were plated and cultured in RPMI with 10% fetal

bovine serum plus L cell media for 7 days as described (Ogawa et al., 2004). Fetal liver-derived macrophages of $TNF^{-/-}cRel^{-/-}$ (control) and $TNF^{-/-}cRel^{-/-}RelA^{-/-}$ ($NF\text{-}\kappa B^{-/-}$) were obtained by mating $TNF^{-/-}cRel^{-/-}RelA^{+/-}$ mice and by genotyping the embryos.

Expression Array Profiling

Cells were lysed with Trizol (Invitrogen) and total RNA was purified using RNeasy columns (Qiagen). cRNA was generated from 10 μg total RNA using Superscript (Invitrogen) and the High Yield RNA transcription labeling kit (Enzo). Fragmented cRNA was hybridized to Affymetrix Mu11 or Codelink mouse Uniset 1 microarrays according to the manufacturer's instructions. Data were analyzed with Microarray Suite (Affymetrix), GeneSpring (Silicongenetics) and in-house software developed as described (Ogawa et al., 2004). Two to four biological replicates were performed for each experimental condition. In addition, results for NR transrepression of LPS signaling were independently validated on both microarray platforms.

Computational Analysis

Proximal promoter regions were extracted for each gene represented on the microarray using the May 2004 mouse genome assembly with the method as described (Halees et al., 2003). Analysis was restricted to the region 1 kb upstream of the transcription start site. In cases were several possible alternative promoters may be present, analysis was focused on the most 5′ transcription start site. Motif discovery was performed using a comparative algorithm previously described (Segal et al., 2002). Promoters were initially divided into two sets: those that were upregulated by LPS and those that were present on the array but did not change in response to LPS. An exhaustive search for all n-mers ($6 < n < 12$) was performed and each n-mer was scored for its enrichment in the promoters upregulated by LPS using the hypergeometric distribution. The top 500 n-mers with a p value less than 0.01 were then clustered together and used to create position-specific probability matrices. The matrices were then further optimized to discriminate between the LPS responsive and nonresponsive genes by the methods as described (Segal et al., 2002).

Transient Transfection and Reporter Studies

Transient transfections were performed as described (Ricote et al., 1998). RAW 264.7 cells were transfected with iNOS promoter luciferase, pCMX-PPARγ and *renilla* luciferase reporter plasmid was also cotransfected as an internal control (Promega). Cells were treated with LPS in the presence of GR, PPAR, and LXR agonists and harvested 24 hr later for analysis of luciferase activity. Double-stranded, short interfering RNAs (siRNA) were synthesized by Dharmacon Research (Lafayette) and were transfected for 48 hr prior to activation with ligands and LPS induction as previously described (Perissi et al., 2004). Data are represented as mean ±SD.

GST Pull-Down Assays

GST pull-down assays were carried out as described previously (Li et al., 2000). GST fusion proteins were produced as crude bacterial lysates and immobilized on glutathione agarose beads. p65, IRF3, full-length GR, GR(K471A), and GR-DBD proteins were translated in vitro using ^{35}S-labeled methionine and TnT-coupled reticulocyte lysate system (Promega).

ChIP Assay

ChIP assay was conducted as previously (Ogawa et al., 2004; Perissi et al., 2004). Anti-IRF3 (Zymed), anti-CBP (Santa Cruz), anti-p65 (Santa Cruz), and anti-GR (Santa Cruz) antibodies were used in immunoprecipitation experiments.

RNA Analysis

RNA analysis by Northern blotting was performed as described (Valledor et al., 2004). Real-time quantitative PCR (SYBRgreen) analysis was performed on an Applied Biosystems 7300 Real-Time PCR system.

LPS-Induced Endotoxin Shock and In Vivo Studies

Combinatorial effects of GR, PPARγ, and LXR agonists in antagonism of LPS responses in vivo were evaluated by measuring TNFα and IL-12 p40 levels 6 and 8 hr after intraperitoneal injection of LPS at 1 mg/mouse, respectively. Mice were orally dosed daily with Dex for 7 days and were intraperitoneally injected with LPS (1 mg/mouse). Blood was collected after LPS stimulation and analyzed for cytokine levels by ELISA. At a minimum, six mice were used for each experimental condition.

Supplemental Data

Supplemental Data include four figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/122/5/707/DC1/.

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