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Parallel SUMOylation-Dependent Pathways Mediate Gene- and Signal-Specific Transrepression by LXRs and PPARγ

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SUMMARY

Transrepression is widely utilized to negatively regulate gene expression, but the mechanisms by which different nuclear receptors effect gene- and signal-specific transrepression programs remain poorly understood. Here, we report the identification of alternative SUMOylation-dependent mechanisms that enable PPARγ and LXRs to negatively regulate overlapping but distinct subsets of proinflammatory genes. Ligand-dependent conjugation of SUMO2/3 to LXRs or SUMO1 to PPARγ targets them to promoters of TLR target genes, where they prevent the signal-dependent removal of NCoR corepressor complexes required for transcriptional activation. SUMO1-PPARγ and SUMO2/3-LXRs inhibit distinct NCoR clearance mechanisms, allowing promoter- and TLR-specific patterns of repression. Mutational analysis and studies of naturally occurring oxysterol ligands indicate that the transactivation and SUMOylation-dependent transrepression activities of LXRs can be independently regulated. These studies define parallel but functionally distinct pathways that are utilized by PPARγ and LXRs to differentially regulate complex programs of gene expression that control immunity and homeostasis.

INTRODUCTION

Members of the nuclear-receptor family of ligand-dependent transcription factors influence immune responses by positively and negatively regulating gene expression in diverse cell types, including macrophages, lymphocytes, and dendritic cells (Glass and Ogawa, 2006; Winoto and Littman, 2002). Recent studies of the glucocorticoid receptor (GR), liver X receptors (LXRs), peroxisome proliferator-activated receptor γ (PPARγ), retinoic acid receptor (RAR), and estrogen receptors (ERs) revealed that each nuclear receptor represses an overlapping, but distinct, pattern of signal-dependent inflammatory gene expression (Galon et al., 2002; Joseph et al., 2003; Ogawa et al., 2005; Welch et al., 2003). Although these observations are consistent with the overlapping but distinct effects of specific ligands on inflammatory processes at a biological level, the molecular mechanisms that are utilized to achieve this specificity remain poorly understood.

LXRα and β are members of the nuclear receptor superfamily that play essential roles in cholesterol and fatty acid homeostasis (Janowski et al., 1996; Peet et al., 1998; Schultz et al., 2000). LXRs are required for normal cholesterol efflux from peripheral cells and cholesterol excretion by the liver by positively regulating the expression of genes involved in cholesterol efflux (Costet et al., 2000; Repa et al., 2000) and genes involved in bile acid biosynthesis and transport (Chiang et al., 2001; Lewis and Rader, 2005). The transcriptional activities of LXRs are thought to be regulated in vivo by oxysterols, oxygenated derivatives of cholesterol (Janowski et al., 1996, 1999).

Recent studies have characterized LXRs as regulators of innate immunity and inflammatory signaling in macrophages (Zelcer and Tontonoz, 2006). LXR agonists enhance macrophage survival during bacterial infection, and LXRs are required for normal immunity to *Listeria monocytogenes* (Castrillo et al., 2003; Joseph et al., 2003, 2004; Valledor et al., 2004). In addition, LXR activation inhibits the lipopolysaccharide (LPS) induction of a number of proinflammatory genes such as inducible nitric oxide synthase (*inos*), cytokines such as interleukin-1β (*il1*β), and chemokines such as monocyte chemoattractant protein-1 (*mcp-1*) (Joseph et al., 2003). Repression

of inflammatory mediators by LXR agonists is abolished in macrophages lacking both LXRs, but not in macrophages containing either LXRα or LXRβ, indicating that both isoforms can mediate anti-inflammatory activity (Joseph et al., 2003). The molecular mechanisms underlying the repression of inflammatory-response genes by LXRs remain to be established.

PPARγ, a nuclear receptor required for fat-cell development and normal glucose homeostasis (Spiegelman, 1998; Willson et al., 2001), also exerts broad anti-inflammatory effects in macrophages and other cell types (Delerive et al., 1999; Glass and Ogawa, 2006; Jiang et al., 1998; Ricote et al., 1998), and this activity has been correlated with insulin-sensitizing and antiatherogenic activities of synthetic PPARγ agonists (Haffner et al., 2002; Li et al., 2000; Staels and Fruchart, 2005). Recent studies of the mechanism by which PPARγ represses activation of the *inos* gene by LPS led to the identification of a SUMOylation-dependent transrepression pathway (Pascual et al., 2005). In this pathway, synthetic PPARγ agonists promote PIAS1-dependent conjugation of SUMO1 to the PPARγ ligand binding domain. This in turn targets PPARγ to NCoR corepressor complexes that are normally bound to the *inos* promoter under basal conditions and are required to mediate active repression in the absence of an inflammatory stimulus. The recruitment of SUMO1-PPARγ was found to prevent the signal-dependent ubiquitylation and clearance of the NCoR complex required for full gene activation, resulting in the *inos* gene remaining in a repressed state (Pascual et al., 2005). These findings raised a series of questions, including whether this pathway is used by other nuclear receptors, whether it can be utilized to mediate repression in a gene or signal-specific manner, and whether natural ligands can promote entry of their cognate nuclear receptors into this pathway.

Here, we demonstrate that LXR transrepression of inflammatory target genes also utilizes a SUMOylation and NCoR-dependent pathway, indicating that this mechanism represents a general molecular strategy for transrepression by a subset of nuclear receptors that regulate metabolism and immunity. In contrast to PPARγ, however, LXRs are SUMOylated by SUMO2 and SUMO3 rather than SUMO1, and HDAC4 rather than PIAS1 functions as a required SUMO E3 ligase. As a consequence, transrepression by PPARγ and LXRs can be independently regulated in a signal- and gene-specific manner.

RESULTS

LXR-Dependent Transrepression Requires NCoR
Based on the requirement of NCoR for transrepression by PPARγ, we initially sought to determine whether LXR-mediated repression is also NCoR dependent. Experiments were performed in primary macrophages transfected with control nonspecific or NCoR-specific siRNAs (Figure S1A in the Supplemental Data available with this article online), using GW3965 as an LXR-specific agonist. Reduction of NCoR expression significantly reversed the

LXR ligand-dependent repression of *inos* expression induced by LPS (Figure 1A). To confirm this result, *inos* promoter activity was evaluated in RAW264.7 macrophages. As shown in Figure 1B, knockdown of NCoR reversed the LXRα and LXRβ-dependent transrepression of *inos* promoter activity induced by LPS. To determine whether LXRs are recruited to the *inos* promoter and prevent NCoR clearance, chromatin immunoprecipitation (ChIP) experiments were performed examining LXR and NCoR occupancy on the *inos* promoter in primary LPS-stimulated macrophages. LXRs were recruited to the *inos* promoter in a ligand-dependent manner in the presence or absence of LPS, and this recruitment was NCoR dependent (Figure 1C). LXRs were not detected in double *lxrα/lxrβ* knockout macrophages, indicating that the LXR antibody specifically detected LXRα/LXRβ bound to the *inos* promoter. As expected, NCoR was found to occupy the *inos* promoter under basal conditions and to be dismissed by LPS (Pascual et al., 2005), whereas treatment with GW3965 prevented NCoR clearance (Figure 1D). These results suggest that LXRα and LXRβ, in addition to PPARγ, repress LPS induction of the *inos* gene by preventing the clearance of NCoR.

LXR-Dependent Transrepression Is SUMOylation Dependent
The identification of LXR transrepression as NCoR dependent raised the question of whether a SUMOylation-dependent pathway was also involved. The ligation of SUMO to target proteins requires the E2-conjugating enzyme Ubc9 (Hay, 2005). We therefore knocked down Ubc9 expression by using a specific siRNA (Figure S1B) in primary macrophages (Figure 2A) and in RAW264.7 cells (Figure 2B) transfected with LXRα and LXRβ expression plasmids. Knockdown of Ubc9 significantly impaired LXR ligand-dependent repression of LPS-induced *inos* expression (Figure 2A) and *inos* promoter activation (Figure 2B). ChIP assays were next performed in primary macrophages to determine the role of SUMOylation in ligand-dependent recruitment of LXRα and LXRβ to the *inos* promoter and in the prevention of NCoR clearance. Knockdown of Ubc9 expression prevented ligand-dependent recruitment of LXR to the *inos* promoter (Figure 2C) and impaired the block in NCoR clearance from the *inos* promoter (Figure 2D). These results suggest that a SUMOylation-dependent step is critical for the recruitment of LXR to the *inos* promoter to prevent corepressor clearance.

LXRs Are Modified by SUMO2/3 Dependent on HDAC4 E3 Ligase Activity
Because a SUMOylation-dependent step is required for LXR transrepression, we tested whether LXR itself was a target of SUMOylation. Flag-tagged LXRβ was expressed in HeLa cells in the presence or absence of Myc-tagged SUMO1, SUMO2, or SUMO3. High-molecular-weight LXRβ, with an increased size expected for monoSUMOylation, was detected in the presence of SUMO2 and SUMO3, but not in the presence of SUMO1
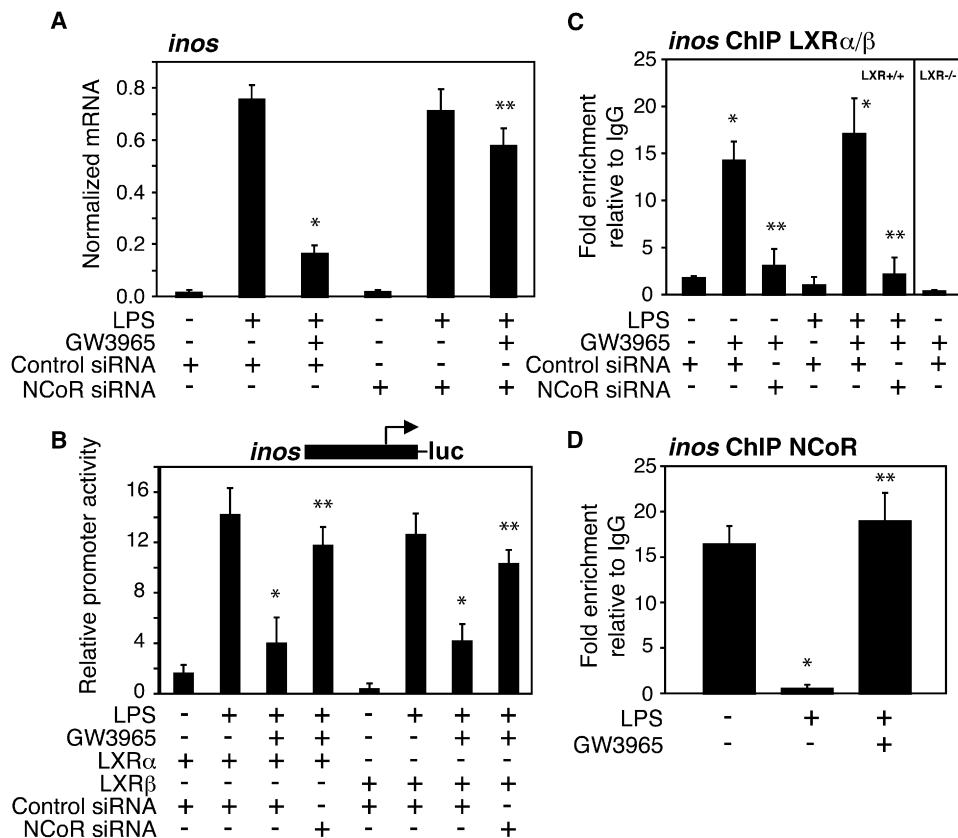
Figure 1. LXR Transrepression Requires NCoR

NCoR-specific siRNA abolishes LXR-dependent repression of LPS-induced endogenous *inos* gene expression in primary macrophages (A) and *inos*-luciferase reporter gene activity in RAW264.7 cells (B). After siRNA transfection, macrophages were stimulated with LPS (1 μg/ml) for 6 hr in the absence or presence of LXR ligand (GW3965, 1 μM). *inos* expression in peritoneal macrophages was evaluated by QPCR, and *inos* promoter activation in RAW264.7 cells was evaluated by luciferase assay. *p < 0.03 versus LPS + control siRNA, and **p < 0.02 versus GW3965 + LPS control siRNA. (C) ChIP assay reveals ligand-dependent and NCoR-dependent LXR recruitment to the *inos* promoter. Primary macrophages were treated with LPS (1 μg/ml) with or without the LXR ligand (GW3965, 1 μM) for 1 hr. ChIP assay was performed with antibody against LXR (both isoforms, α and β) or control IgG. Immunoprecipitated DNA was analyzed by QPCR using primers specific for the *inos* promoter. Chromatin isolated from *lxr*$^{-/-}$ mice was used as a negative control for specificity of the LXR antibody. *p < 0.05 versus untreated or LPS alone, and **p < 0.03 versus GW3965 or GW3965 + LPS control siRNA. (D) LXR ligand inhibits LPS-stimulated release of NCoR from the *inos* promoter as shown by ChIP assay in peritoneal macrophages, treated as described in (C). *p < 0.02 versus untreated, and **p < 0.02 versus LPS. Results are expressed as the average of three independent experiments. Error bars represent standard deviations.

(Figure 3A) in a ligand-dependent manner. This high-molecular-weight species was detected by anti-Myc antibody and was abolished by knockdown of Ubc9 (Figure 3B), indicating that it corresponds to SUMOylated LXRβ. LXRα was also SUMOylated by SUMO2 and SUMO3 (data not shown). An additional LXR-dependent band indicated by asterisks in Figure 3 was also observed in some experiments. This band likely represents an unknown posttranslational modification of LXR that is not correlated with SUMOylation.

Several SUMO E3 ligases have been identified that promote transfer of SUMO from the E2 to specific substrates. To date, three types of SUMO E3 ligases have been well characterized: the protein inhibitor of activated signal transducer and activator of transcription (PIAS) family, the RanBP2/Nup358 protein, and the human Polycomb member Pc2 (Melchior et al., 2003). Although SUMO-PPARγ-mediated transrepression is PIAS1 dependent (Pascual et al., 2005), the systematic knockdown of all PIAS E3 ligases, RanBP2/Nup358, and PC2 did not reverse LXR ligand-dependent repression in primary macrophages (data not shown). Recently, HDAC4 and related members of class II deacetylases, such as HDAC5, have been described as SUMO E3 ligases for MEF2 transcription factor (Gregoire and Yang, 2005; Zhao et al., 2005). Knocking down HDAC4 expression (Figure S1C) resulted in a complete inhibition of LXRβ SUMOylation (Figure 3C). Consistent with these findings, a physical interaction between LXRβ and HDAC4 was revealed by coimmunoprecipitation assays (Figure 3D). To determine whether HDAC4 directly promotes LXRs SUMOylation, we performed an in vitro SUMOyation assay. Ligand-dependent
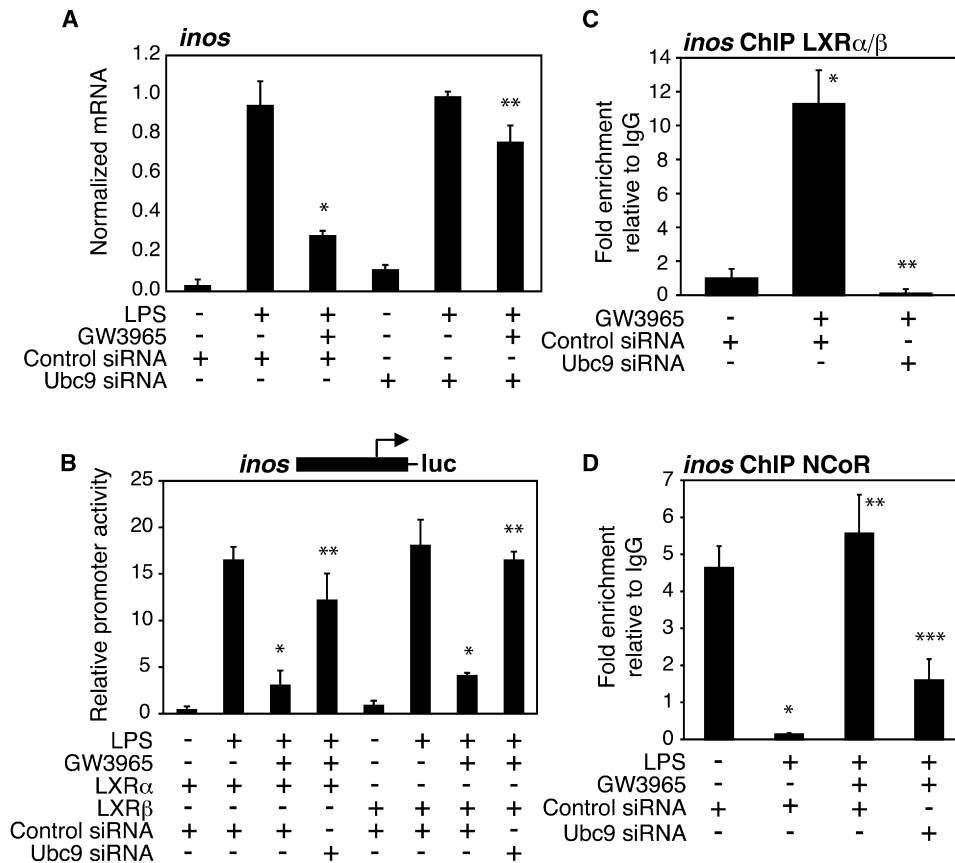
Figure 2. LXR-Dependent Transrepression Is SUMOylation Dependent

Ubc9-specific siRNA reverses LXR-dependent repression of LPS-induced *inos* expression in primary macrophages (A) and *inos* promoter activation in RAW264.7 cells (B). After siRNA transfection, macrophages were stimulated with LPS (1 μg/ml) for 6 hr in the absence or presence of LXR ligand (GW3965, 1 μM). *inos* expression in peritoneal macrophages was evaluated by QPCR, and *inos* promoter activation in RAW264.7 was evaluated by luciferase assay. *p < 0.03 versus LPS control siRNA, and **p < 0.02 versus GW3965 + LPS control siRNA. (C and D) siRNA directed against Ubc9 prevents LXR recruitment (C) and abolishes the ability of the LXR agonist to inhibit the NCoR clearance (D) from the *inos* promoter as shown by ChIP. Primary macrophages were treated with LPS (1 μg/ml) with or without the LXR ligand (GW3965, 1 μM) for 1 hr. ChIP assay was performed with antibody against LXR or NCoR or control IgG. Immunoprecipitated DNA was analyzed by real-time PCR using primers specific for the *inos* promoter. (C) *p < 0.01 versus untreated + control siRNA; **p < 0.02 versus GW3965 control siRNA. (D) *p < 0.02 versus untreated + control siRNA, **p < 0.02 versus LPS control siRNA, and ***p < 0.01 versus GW3965 + LPS control siRNA. Results are expressed as average of two independent experiments. Error bars represent standard deviations.

LXRβ SUMOylation was significantly increased by the addition of in vitro-translated HDAC4 protein in the presence of ligand (Figure 3E), but not in the absence of ligand (data not shown). In contrast, an HDAC4 mutant (67-257), which does not have the coiled-coil domain required for the interaction with Ubc9 (Zhao et al., 2005), abolished LXRβ SUMOylation. Similar effects were observed in HeLa cells overexpressing WT HDAC4 or the HDAC4 mutant (67-257) (Figure 3F). In concert, these findings strongly suggest that HDAC4 functions as a SUMO E3 ligase for LXRs in vivo.

We next evaluated whether HDAC4 was required for LXRs-mediated transrepression in primary macrophages. Unexpectedly, knockdown of HDAC4 prevented LPS activation of *inos*, precluding an assessment of its role in transrepression of this specific target (data not shown). Several

recent reports suggest that activation of inflammatory response genes by IRFs is blocked by HDAC inhibitors (Chang et al., 2004; Nusinzon and Horvath, 2003; Sakamoto et al., 2004). As the *inos* gene is also a target of these factors, we speculate that HDAC4 is one of the key HDACs that are required for activation of this cohort of genes.

To identify alternative LPS target genes that would be suitable for determining the role of HDAC4 in LXR-dependent transrepression, microarray experiments were performed to identify LPS-inducible, LXR-sensitive target genes that retained LPS-induction in the presence of the HDAC inhibitor TSA (Table S1). *mcp-1* was chosen as one of the most representative chemokine genes meeting these criteria. To determine whether the *mcp-1* gene was an appropriate model, we performed transrepression experiments in primary macrophages and confirmed that
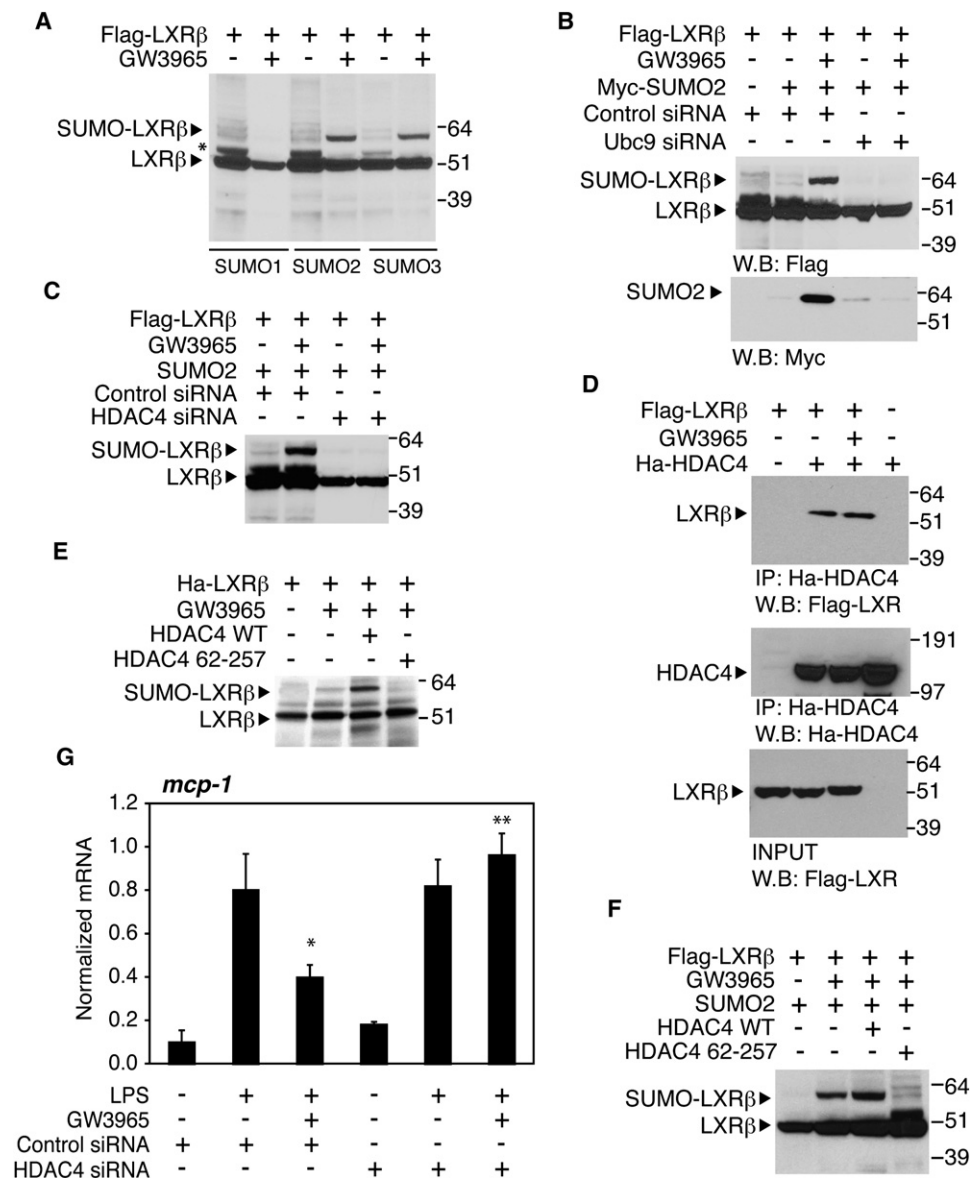
Figure 3. LXRβ Is Modified by SUMO2 and SUMO3

(A) LXRβ is SUMOylated by SUMO2 and SUMO3. HeLa cells were transfected with Flag-LXRβ, Ubc9, and with SUMO1, SUMO2, or SUMO3 expression vector (as indicated) and treated with GW3965 for 1 hr (1 μM). Whole-cell lysates were immunoblotted for Flag tag.

(B) Knockdown of Ubc9 by specific siRNA blocks the SUMO2 conjugation of LXRβ. HeLa cells were transfected with siControl, siUbc9 (where indicated), Flag-LXRβ, and MYC-SUMO2 expression vectors. Whole-cell lysates were immunoprecipitated with Flag antibody and immunoblotted for Flag tag and MYC-SUMO2 tag.

(C) An siRNA directed against HDAC4 blocks LXRβ SUMOylation. HeLa cells were transfected with siControl, siHDAC4 (where indicated), Flag-LXRβ, and SUMO2 expression vectors and treated with GW3965 for 1 hr (1 μM). Whole-cell lysates were immunoblotted for Flag tag.

(D) LXRβ and HDAC4 physically interact. HeLa cells were transfected with Flag-LXRβ and Ha-HDAC4 and treated with GW3965 for 1 hr as indicated. Whole-cell lysate was immunoprecipitated with HA antibody (middle), and LXRβ was detected with Flag antibody (top). In the bottom panel, the INPUT shows an equal expression of LXRβ in each sample. Cells transfected with only LXRβ or HDAC4 (first and last lane) were used as negative control for the IP.

(E) LXRβ is SUMOylated by HDAC4 in vitro. The in vitro SUMOylation assay was performed as described in the Experimental Procedures, adding to the reaction HDAC4 or HDAC4 mutant (67-257) as indicated. The proteins were immunoblotted for HA tag.

(F) HDAC4 modulates LXRβ SUMOylation in vivo. HeLa cells were transfected with Flag-LXRβ, Ubc9, SUMO2, and HDAC4 or HDAC4 mutant (67-257) expression vector (as indicated) and treated with GW3965 for 1 hr (1 μM). Whole-cell lysates were immunoblotted for Flag tag.

(G) Knockdown of HDAC4 blocks LXR transrepression of *mcp-1*. Primary macrophages were transfected with control or HDAC4-specific siRNA for 48 hr and then stimulated with LPS (1 μg/ml) for 6 hr in the absence or presence of LXR ligand (GW3965, 1 μM). *mcp-1* expression was evaluated by real-time PCR. Results are expressed as average of two independent experiments. Error bars represent standard deviations. * p < 0.02 versus LPS + control siRNA, and **p < 0.03 versus GW3965 + LPS + control siRNA.

mcp-1 transrepression was NCoR and Ubc9 dependent (Figure S2). ChIP assays confirmed that LXRs were recruited to the *mcp-1* promoter in an NCoR and Ubc9-dependent manner and prevented NCoR clearance (Figures S3A and S3B). Knockdown of HDAC4 (Figure S1C) had no effect on induction of *mcp-1*, as predicted by the microarray data, but reversed LXR transrepression (Figure 3G). Taken together, these results demonstrate that HDAC4 interacts with LXR and regulates LXR transrepression activity by promoting its SUMOylation. Furthermore, these findings indicate that, although both LXR and PPARγ transrepression are SUMOylation dependent, LXR is SUMOylated in a ligand-dependent manner in vivo by SUMO2 and SUMO3, whereas PPARγ is modified by SUMO1 (Floyd and Stephens, 2004; Ohshima et al., 2004; Pascual et al., 2005; Yamashita et al., 2004).

Mutation of LXR SUMO Acceptor Sites Abolishes Transrepression, but Not Transactivation Activity

Two lysine residues in the human LXRβ primary amino acid sequence, the N-terminal K31 and the C-terminal K448, conform with high probability to the proposed consensus motif for SUMO conjugation. In addition, a lower-probability site was identified at K410. To determine whether these lysine residues are targets for SUMOylation, mutants with lysine to arginine substitutions of K31, K410, and K448 were generated and assayed for SUMOylation. SUMOylation of LXRβ was reduced when the cells were transfected with mutants K410 and K448 and completely abolished with K410/448 double mutation, whereas mutation of K31 had no effect (Figure 4A).

To determine the functional significance of LXR SUMOylation, the abilities of the LXRβ mutants to transrepress the *inos* promoter and transactivate the *abca1* promoter were tested in RAW264.7 cells. Individual mutations of K410 and K448 partially impaired transrepression activity, whereas the K410/448 double mutant fully abolished transrepression (Figure 4B). Similar results were observed for the *mcp-1* promoter (Figure S3C). In contrast, these same lysine mutations did not impair ligand-dependent transactivation of the *abca1* promoter. Parallel studies of LXRα demonstrated ligand-dependent SUMOylation of lysine residues 328 and 434 (corresponding to residue 448 of LXRβ). Mutation of these residues to arginine impaired LXRα transrepression, but not transactivation (data not shown). In concert, these experiments indicate that specific lysine residues in the ligand binding domains of LXRα and LXRβ are SUMOylated by SUMO2 and SUMO3 and that their SUMOylation is required for transrepression.

Endogenous LXR Ligands Promote Entry into the Transrepression Pathway

A number of naturally occurring oxysterols are potent transcriptional activators of LXRs (Janowski et al., 1996, 1999). In macrophages, endogenous oxysterols are thought to be produced in proportion to intracellular cholesterol levels and induce the expression of a number of
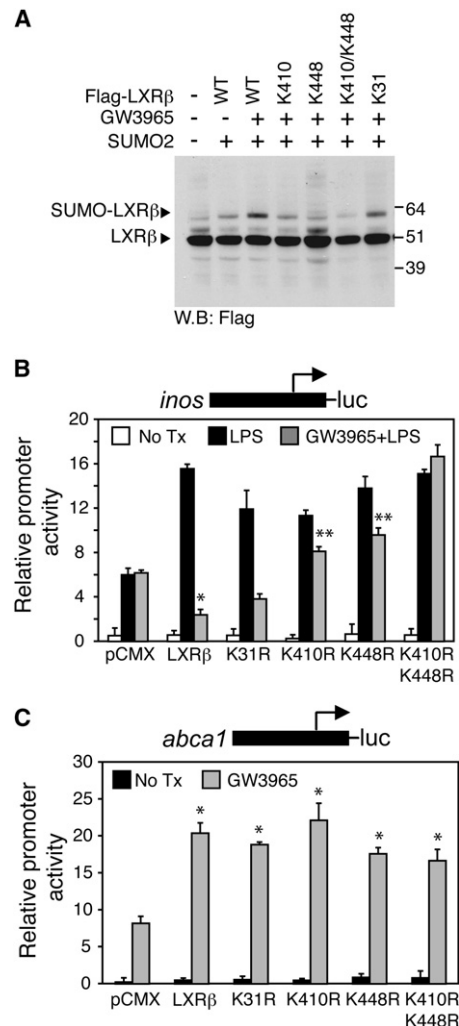

Figure 4. Mutation of LXRβ SUMO Acceptor Sites Abolishes Transrepression, but Not Transactivation Activity

(A) SUMOylation of LXRβ occurs at K410 and K448, but not at K31, as demonstrated by SUMOylation assay (performed as described in Figure 3A) in HeLa cells transfected with WT Flag-LXRβ or Flag mutants K31, K410, and K448 or double mutants K410/K448 as indicated. (B and C) LXRβ K410 and K448 are required for transrepression, but not for transactivation. RAW264.7 cells were transfected with *inos*-luc (B) or *abca1*-luc (C) reporter plasmids and with an expression vector for LXRβ wild-type or mutated in K31, K410, K448, or K410/K448 as indicated. Twenty-four hours after transfection, cells were treated with DMSO (white bars) or with LPS (1 μg/ml) for 12 hr in the absence (black bars) or presence of LXR ligand (GW3965, 1 μM, gray bars). Results are expressed as average of three independent experiments. Error bars represent standard deviations. (B) *p < 0.01 versus LPS, and **p < 0.05 versus GW3965 + LPS + LXRβ wild-type . (C) *p < 0.03 versus pCMX.

genes involved in LXR-dependent cholesterol efflux, such as *abca1* (Repa et al., 2000). To investigate the relative abilities of naturally occurring LXR ligands to both positively and negatively regulate gene expression, their effects on *abca1* gene expression and LPS-dependent
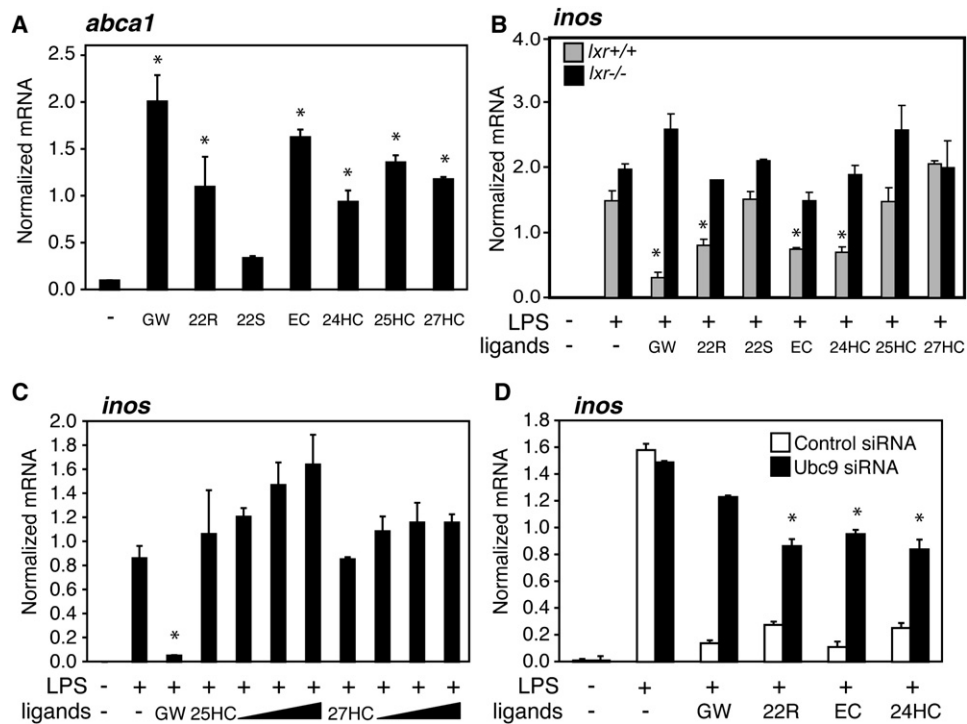
Figure 5. Endogenous LXRs Ligands Promote Entry into Transrepression Pathway

(A and B) Natural LXRs ligands induce *abca1* expression and repress *inos* activation. Primary macrophages from WT ([A] and [B], gray bars) and *lxr*^{−/−} mice ([B], black bars) were treated with GW3965 (1 μM) or 5 μM oxysterols 22R, 22S, 24(S),25EC, 24HC, 25HC, and 27HC (A) for 18 hr. In (B), cells were pretreated with the indicated ligands and then treated with LPS (1 μg/ml) for 6 hr. Expression of *abca1* (A) and *inos* (B) was evaluated by QPCR. (A) *p < 0.03 versus untreated; (B) *p < 0.01 versus LPS.

(C) Dose-response experiment of 25HC and 27HC. Primary macrophages were pretreated with GW3965(1 μM), 25HC, and 27HC at increasing concentrations (1 μM, 5 μM, 10 μM, and 50 μM) and then treated with LPS (1 μg/ml) for 6 hr. Expression of *inos* was evaluated by QPCR. *p < 0.01 versus LPS.

(D) Natural LXR ligands require Ubc9 to transrepress *inos* expression. Primary macrophages were transfected with Ubc9 siRNA (black bars) or non-specific siRNA (white bars) for 48 hr and then stimulated with LPS (1 μg/ml) for 6 hr in the absence or presence of LXR ligands as indicated (5μM). *inos* expression in peritoneal macrophages was evaluated by QPCR. *p < 0.05 versus control siRNA. Results are expressed as average of two independent experiments. Error bars represent standard deviations.

induction of the endogenous *inos* gene were evaluated in primary macrophages. These experiments revealed that 22(R)-hydroxycholesterol (22R), 24(S),25 epoxycholesterol (EC), and 24-hydroxycholesterol (24HC) each repressed *inos* activation and induced *abca1* expression. This anti-inflammatory effect was LXR dependent, as it was not observed in *lxr*^{−/−} macrophages (Figure 5B). In contrast, 25-hydroxycholesterol (25HC) and 27-hydroxycholesterol (27HC) were able to activate *abca1* but did not repress *inos* activation even at significantly higher concentrations than those that allow activation of the *abca1* gene (Figure 5C), indicating that activation and transrepression activities can be chemically separated. Furthermore, the transrepression activities of 22R, 24(S), 25-epoxycholesterol (EC), and 24HC are impaired by knocking down Ubc9, suggesting that a subset of the naturally occurring oxysterol ligands of LXRs can promote their entry into the SUMOylation-dependent transrepression pathway.

Gene-Specific Utilization of Parallel NCoR and SUMOylation-Dependent Pathways by LXRs and PPARγ

The finding that a SUMOylation/NCoR-dependent pathway parallel to that initially identified to mediate transrepression of inflammatory responses by PPARγ is utilized by LXRs to repress *inos* activation raised the questions of the extent to which these parallel pathways are utilized to repress other LPS target genes and whether they can be utilized in a gene-specific manner. Previous expression profiling studies demonstrated that PPARγ and LXR agonists repressed overlapping but distinct sets of genes induced in primary macrophages by LPS or the TLR3-activator poly I:C (Ogawa et al., 2005). These relationships are illustrated in Figure 6A, filtered for genes induced at least 5-fold for each TLR agonist, and exhibiting at least 50% repression with respect to activation in the absence of PPARγ or LXR agonist. We initially evaluated several endogenous LPS-inducible target genes that were found
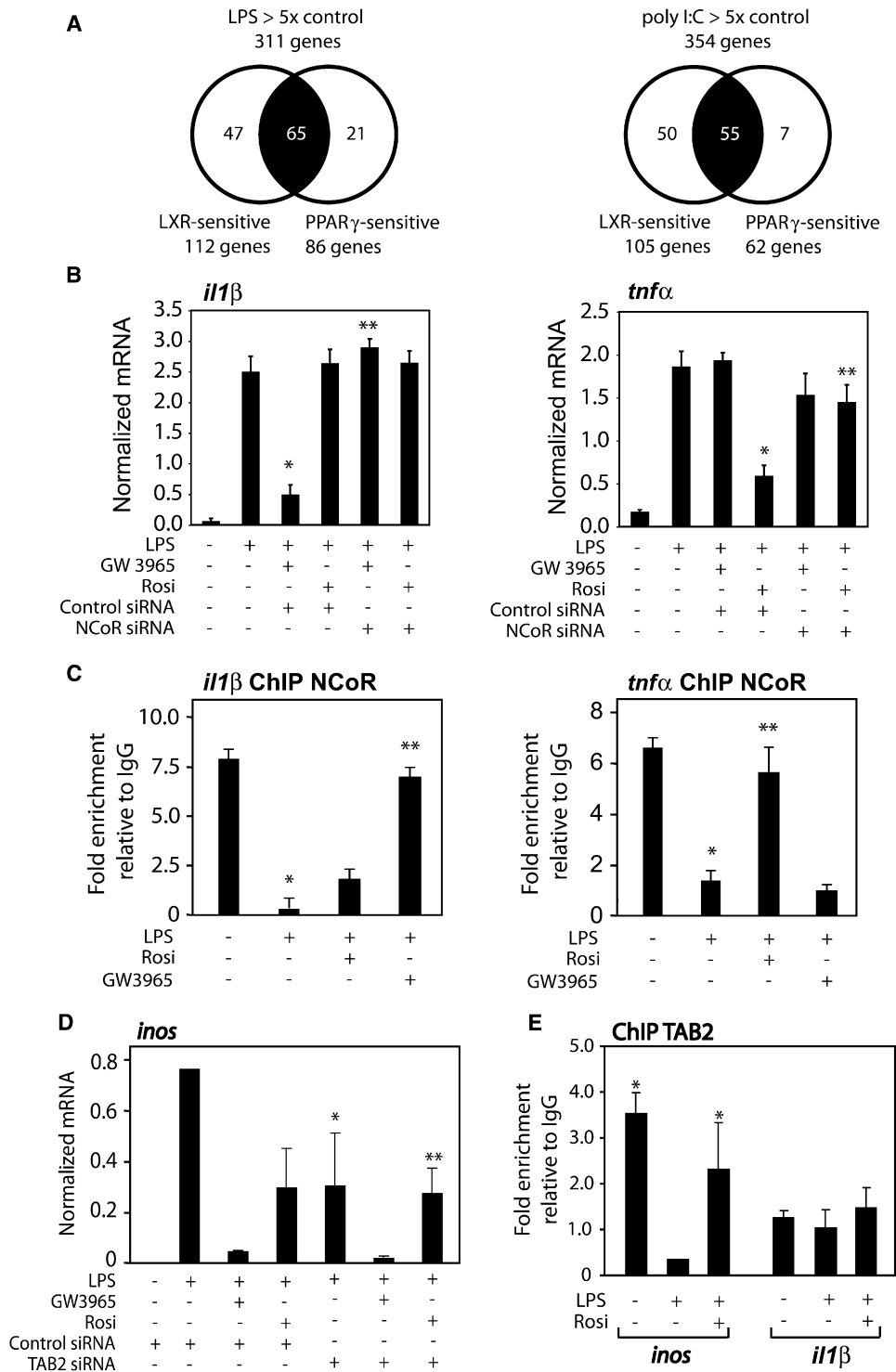
Figure 6. Gene-Specific Utilization of NCoR and SUMOylation-Dependent Pathways in LXRs and PPARγ Transrepression

(A) Relationship of genes subject to transrepression by LXR or PPARγ agonists in primary macrophages stimulated with either LPS or poly I:C as determined by expression profiling assays. Genes were filtered based on at least 5-fold induction by LPS and poly I:C and at least 50% repression by each PPARγ or LXR agonist. Data is derived from Ogawa et al. (2005).

(B) Receptor-specific repression of *il1β* and *tnfα*. Primary macrophages were transfected with siRNA control or NCoR-specific siRNA for 48 hr and then stimulated with LPS (1 μg/ml) for 6 hr in the absence or presence of LXR ligand (GW3965, 1 μM) or PPARγ ligand (Rosiglitazone, 1 μM). *il1β* and *tnfα* expression was evaluated by QPCR. *p < 0.01 versus LPS, and **p < 0.01 versus GW3965 (left) or Rosi (right) + LPS control siRNA.

to be transrepressed by both nuclear receptors in primary macrophages. The majority of the LPS-inducible genes meeting these criteria, exemplified by *mip-1β*, *mcp-1*, *vcam-1*, and *il-15*, were found to be transrepressed by PPARγ and LXRs by an NCoR and SUMOylation-dependent mechanism (Figure S2). These findings indicate that the NCoR and SUMOylation-dependent pathways are likely to be widely utilized by LXRs and PPARγ to transrepress proinflammatory target genes.

We next evaluated subsets of LPS-inducible genes that were transrepressed only by the LXR agonist (represented in Figure 6B by *il1β*) or only by the PPARγ agonist (represented in Figure 6B by *tnfα*). Unexpectedly, although *il1β* and *tnfα* were differentially transrepressed by LXRs and PPARγ ligands, both cases involved an NCoR-dependent transrepression mechanism (Figure 6B). This result was confirmed by ChIP experiments, showing that the LXR agonist, but not the PPARγ agonist, was able to inhibit the clearance of NCoR from *il1β* promoter (Figure 6C, left). Conversely, NCoR was cleared from the *tnfα* promoter in the presence of the LXR agonist, but not after treatment with the PPARγ agonist (Figure 6C, right). We therefore performed ChIP experiments in primary macrophages to determine whether the SUMOylated receptors were differentially recruited to these promoters. These assays indicated that PPARγ and LXRs were both recruited to the *inos*, *il1β*, and *tnfα* promoters in a ligand-dependent manner (Figure S4A), suggesting that the SUMOylated receptors are interrupting distinct signaling inputs that are responsible for NCoR clearance. This finding is supported by the observation that saturating concentrations of PPARγ and LXR agonists exerted additive repressive effects on the *inos* promoter, which is sensitive to transrepression by both receptors, suggesting that they operate through independent mechanisms (Figure S4B).

To explore the roles of specific signaling adaptor proteins in LPS activation and transrepression, we investigated TAB2, which plays a cytoplasmic role in TLR4-dependent signal transduction and is also a component of a subset of NCoR complexes (Baek et al., 2002; Jiang et al., 2004; Takaesu et al., 2000; Zhu et al., 2006). Knockdown of TAB2 in macrophages (Figure S1D) reduces the fold activation of *inos* in response to LPS by about 50%. Intriguingly, the residual activation of *inos* remained fully sensitive to repression by LXRs agonist but was now completely resistant to repression by the PPARγ agonist (Figure 6D). Furthermore, ChIP experiments demonstrated that TAB2 was present on the *inos* promoter under basal conditions, was cleared in response to LPS treatment,

but was retained in the presence of LPS and PPARγ agonist. In contrast, TAB2 was not enriched above background (IgG control) levels on the *il1β* promoter under any condition. In concert, these results are consistent with TAB2 playing a promoter-specific role in mediating PPARγ-dependent, but not LXR-dependent, transrepression.

Signal-Specific Utilization of Parallel NCoR and SUMOylation-Dependent Pathways by LXRs and PPARγ

Recent studies demonstrated that the ability of a particular nuclear receptor to repress specific subsets of inflammatory-response genes could be modulated in a signal-specific manner, e.g., a cohort of genes that was sensitive to GR-mediated repression when induced by LPS was GR resistant when activated by poly I:C (Ogawa et al., 2005). A similar relationship was observed for genes subject to transrepression by LXR and PPARγ agonists, illustrated in Figure 7A. To investigate whether genes that exhibit signal-specific repression by LXRs and PPARγ utilize an NCoR-dependent mechanism, we evaluated the *il1β* gene. Although this gene is strongly repressed by LXR agonists in an NCoR-dependent manner when activated by LPS, it is almost completely resistant when activated by poly I:C (Figure 7B). As a second example, we evaluated repression of the *inos* gene when activated by the TLR1/2 agonist Pam3CSK (Omueti et al., 2005).

Although repressed by both LXR and PPARγ agonists when induced by LPS, the ability of the LXR agonist to transrepress *inos* was selectively abolished when macrophages were stimulated by the TLR1/2 agonist (Figure 7C). We therefore evaluated whether the ability of LXR agonist to transrepress inflammatory responses is correlated with their ability to block NCoR clearance induced by different TLR agonists. ChIP experiments revealed that LXR agonists inhibited NCoR clearance from the *inos* promoter in response to LPS treatment but failed to inhibit NCoR clearance when cells were treated with the TLR1/2 agonist Pam3CSK (Figure S5A). In addition, poly I:C was found to promote clearance of NCoR from the *il1β* promoter even when the cells were treated with LXRs ligand, consistent with the failure of GW3965 to transrepress *il1β* activation in response to this signal (Figure S5B). In concert, these results indicate that the parallel SUMOylation-dependent transrepression pathways are themselves subject to regulation and can be overridden by specific signals in a gene-specific manner.

(C) Differential inhibition of NCoR clearance from *il1β* and *tnfα* promoters by PPARγ and LXR ligands. Primary macrophages were treated with LPS (1 μg/ml) with or without PPARγ (Rosiglitazone, 1 μM) or LXR ligand (GW3965, 1 μM) for 1 hr. ChIP assays were performed with antibody against NCoR or control IgG. *p < 0.01 versus untreated, and **p < 0.08 versus LPS.

(D) PPARγ transrepression is TAB2 dependent. Primary macrophages were transfected with siRNA control or TAB2-specific siRNA for 48 hr and then stimulated with LPS (1 μg/ml) for 6 hr in the absence or presence of LXR ligand (GW3965, 1 μM) or PPARγ ligand (Rosiglitazone, 1 μM). *inos* expression was evaluated by QPCR. *p < 0.01 versus LPS, and **p < 0.01 versus Rosi + LPS control siRNA.

(E) TAB2 is present in the basal state on the *inos* promoter, but not on the *il1β* promoter. ChIP assays were performed with antibody against TAB2 or control IgG. *p < 0.01 versus control IgG. Results are expressed as the average of two independent experiments. Errors bars represent standard deviations.
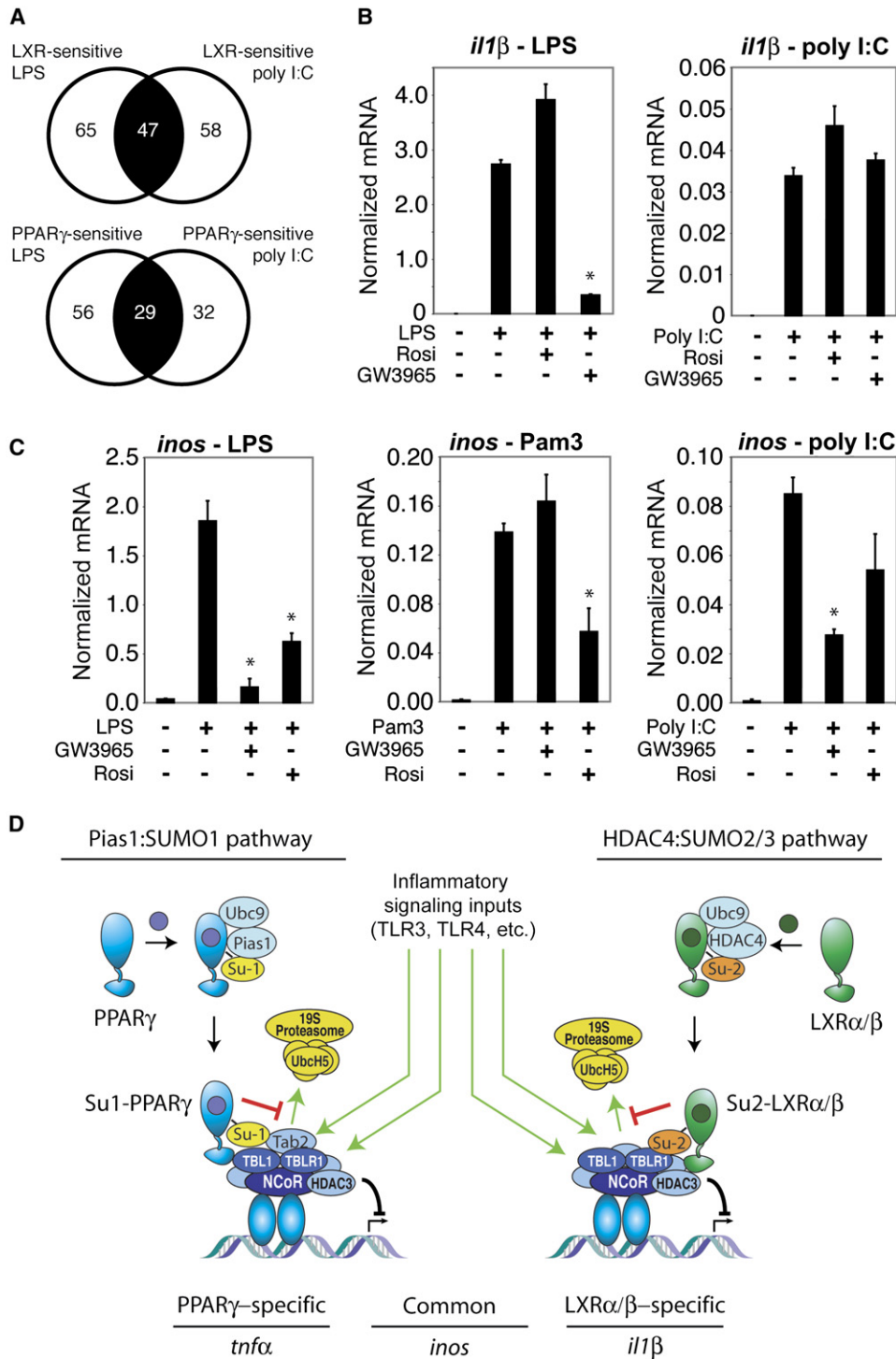
Figure 7. Signal-Specific Regulation of NCoR and SUMOylation-Dependent Transrepression

(A) Signal dependence of repression by PPARγ and LXR agonists. Genes in the central sector of each diagram were repressed when induced by either LPS or poly I:C. Genes in the left sector were only repressed when induced by LPS, whereas genes in the right sector were only repressed when induced by poly I:C.

(B) The *il1*β gene is induced by both LPS and poly I:C but is only sensitive to LXR repression when induced by LPS. Primary macrophages were treated with TLR4-specific agonist (LPS,1 µg/ml) or TLR3-specific agonist (polyI:C, 50 ng/ml) for 6 hr with or without PPARγ (Rosiglitazone, 1 µM) or LXR ligand (GW3965, 1 µM). *il1*β expression was evaluated by QPCR. *p < 0.02 versus LPS.

DISCUSSION

A General SUMO/NCoR Transrepression Mechanism

Many nuclear receptors can act in *trans* to repress inflammatory responses, with recent studies indicating that ligand-dependent repression of inflammatory target genes occurs in a gene and signal-specific manner (Ogawa et al., 2005). Although a large number of transrepression mechanisms have been suggested from the analysis of artificial and transiently transfected target genes, relatively few mechanisms have been established for endogenous target genes in primary cells. The present studies demonstrate that ligand-dependent transrepression of a number of endogenous inflammatory response genes in primary macrophages by LXRs is NCoR- and SUMOylation-dependent. Moreover, ChIP analysis revealed that LXRs are recruited to the *inos* promoter in a SUMOylation-dependent manner and inhibit corepressor clearance induced by LPS. These observations extend the NCoR and SUMOylation-dependent pathway, initially identified for PPARγ (Pascual et al., 2005), to two additional members of the nuclear receptor family: LXRα and LXRβ. Thus, SUMOylation-dependent targeting of a nuclear receptor to corepressor complexes to prevent their signal-dependent clearance is likely to represent a general molecular strategy for transrepression of proinflammatory target genes, expanding upon previously described mechanisms by which SUMO proteins function to negatively regulate gene expression (Gill, 2005).

Parallel SUMO-Dependent Transrepression Pathways

Although these studies indicate that LXRs and PPARγ both utilize a SUMOylation-dependent mechanism that converges on NCoR corepressor complexes, they also provide evidence for distinct and parallel pathways that are used in a receptor-specific and gene-specific manner (Figure 7). These conclusions are based on the findings that PPARγ and LXRs are conjugated to different SUMO proteins by different E3 ligases and differentially regulate proinflammatory target genes such as *il1β* and *tnfα*. Although PIAS1 has been described as the E3 ligase involved in PPARγ SUMOylation (Ohshima et al., 2004; Pascual et al., 2005), the present studies strongly suggest that SUMOylation of LXRs requires HDAC4 E3 ligase activity. In the case of PPARγ, a ligand-induced allosteric change in the position of the lysine residue that is the point of SUMO1 conjugation was proposed to be the basis for ligand-dependent SUMOylation (Pascual et al., 2005). In the case of LXRs, crystal structures of apo receptors are not available, so it is not yet possible to assess ligand-dependent changes in the lysine residues that represent

the essential SUMO acceptor sites. However, association of LXRβ with HDAC4 does not appear to be ligand dependent, suggesting that ligands alter the specific configurations of the lysine residues that serve as SUMO acceptor sites to provide the mechanism for ligand dependence.

Because the conjugated forms of SUMO2 and SUMO3 form a distinct subfamily and are only 50% identical in sequence to SUMO1 (Hay, 2005), we propose that LXRs and PPARγ are functionally differentiated based on their differential SUMOylation and represent distinct, parallel transrepression pathways that can be utilized in a gene and signal-specific manner (Figure 7D). Gene-specific utilization of these parallel pathways is exemplified by the selective transrepression of *il1β* by LXRs and *tnfα* by PPARγ, whereas signal-specific utilization is exemplified by the differential sensitivities of the *il1β* and *inos* genes to LXR ligands when activated by TLR1/2, TLR3, and TLR4 agonists.

These findings thus raise a number of intriguing possibilities regarding the molecular targets of SUMOylated PPARγ and LXRs within the NCoR complex and how the docking of these proteins prevents NCoR clearance. ChIP experiments have now localized NCoR complexes on nearly all LPS-responsive genes we have examined. Furthermore, it appears that SUMOylated PPARγ and LXRs localize to these complexes prior to an activating signal. In all cases, whether or not a gene is sensitive or resistant to PPARγ or LXR agonists is strictly correlated with whether or not they prevent NCoR clearance. Previous studies suggest that signal-dependent clearance of NCoR complexes from target promoters involves the activation of the ubiquitin E3 ligase activities of the Tbl1 and TblR1 proteins that are core components of these complexes (Ogawa et al., 2004; Perissi et al., 2004), leading to the recruitment of the Ubc5/19S proteosome machinery required for ubiquitylation of NCoR complexes and their subsequent dismissal. SUMOylation of PPARγ and its interaction with the NCoR complex have been shown to block signal-dependent recruitment of UbcH5 to the *inos* promoter (Pascual et al., 2005), but whether this is due to inhibition of Tbl1/TblR1 phosphorylation or some distal step remains to be defined. The finding that TAB2 is required for transrepression of the *inos* gene by PPARγ, but not LXRs, suggests that the SUMOylated receptors inhibit distinct signaling inputs that are involved in activating the ubiquitin-ligase activities of Tbl1/TblR1. Biochemical approaches have recently identified components of corepressor complexes as interacting targets of SUMO2 (Rosendorff et al., 2006), and it will be of interest to utilize SUMOylated LXRs and PPARγ as molecular probes to further elucidate the specific events that allow NCoR

(C) Differential transrepression of the *inos* gene by LXR and PPARγ ligands. Primary macrophages were treated with the LPS (1 μg/ml), TLR2-specific agonist (PAM3, 300 ng/ml), or poly I:C (50 ng/ml) for 6 hr with or without PPARγ (Rosiglitazone, 1 μM) or LXR ligand (GW3965, 1 μM). *inos* expression was evaluated by real-time PCR. *p < 0.02 versus LPS (left), Pam 3 (middle), and poly I:C (right). Results are expressed as the average of two independent experiments performed in duplicate. Error bars represent standard deviations.
(D) Model depicting parallel transrepression pathways utilized by PPARγ and LXRs. See text for discussion.

corepressor complexes to be cleared from inflammatory response genes in a signal-specific manner.

Coordinate and Independent Regulation of Transactivation and Transrepression

In macrophages, LXRs play critical roles in maintaining normal cholesterol homeostasis by positively regulating the expression of genes involved in cholesterol storage and efflux. The observation that synthetic LXR agonists are also capable of potently repressing inflammatory response genes raises the question of whether the production of endogenous oxysterols might also exert anti-inflammatory effects by binding to LXRs. Consistent with this possibility, we find that 22R, 24(S),25EC, and 24HC can both induce *abca1* expression and repress *inos* activation in primary macrophages. Intriguingly, 25HC and 27HC can activate *abca1* but do not repress *inos* activation. These findings suggest that the transactivation and transrepression activities of LXRs may be independently regulated based on the specific ligands that are produced under different contexts. Intriguingly, the oxysterols that induce transrepression are not found at any significant levels in cultured macrophages, whereas the oxysterols that are made at high levels by cultured macrophages do not induce transrepression (Fu et al., 2001). It is possible that enzymes necessary for producing natural ligands that activate this pathway are expressed in specific subsets of macrophages in vivo. Alternatively, this pathway may operate as a paracrine system, e.g., Kuppfer cells in liver may be exposed to hepatocyte-derived oxysterols that activate the transrepression pathway.

The ability of many ligand-dependent nuclear receptors to inhibit inflammatory gene expression is also of considerable interest from the perspective of the development of new approaches to modulate inflammatory diseases. This goal is often complicated by the fact that potent nuclear receptor agonists are associated with unwanted side effects: e.g., PPARγ agonists promote weight gain and edema formation in diabetic patients (Staels and Fruchart, 2005; Yki-Jarvinen, 2005), whereas LXR agonists have been shown to cause marked hypertriglyceridemia in animals, at least in part due to activation of SREBP1c in liver (Schultz et al., 2000). The present studies suggest that it should be possible to develop LXR ligands that separate transrepression from transactivation activities. Ligands that specifically promote entry of LXR into the SUMO-dependent transrepression pathway may be of therapeutic utility in diseases in which inflammation plays a significant pathogenic role.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids

TLR4 agonist (LPS) and TLR3 agonist (poly I:C) were obtained from Sigma, TLR1/2 agonist (Pam3CysSK4) from ECM Microcollections, and GW3965 and Rosiglitazone were provided by GlaxoSmithKline. 22R, 22S, and 25HC were purchased from Sigma, 24(S), 25EC, and 24HC from Biomol, and 27HC from Research Plus, Inc. Point mutations in Flag-hLXRα and Flag-hLXRβ were made by using the Quik-

Change side-directed mutagenesis kit (Stratagene). The reporter plasmid *inos*-luc, Ubc9, and SUMO1 expression vectors have been previously described (Pascual et al., 2005), the reporter plasmid *abca1*-luc was kindly donated by Dr. B. Wagner, the reporter plasmid *mcp-1*-luc was kindly donated by Dr. R. Natarajan, and the HDAC4 mutant (67-257) was kindly donated by Dr. T. Yao.

Cell Culture and Transient Transfection

Thioglycollate-elicited macrophages were prepared as previously described (Pascual et al., 2005) from *lxrαβ*+/+ and *lxrαβ*−/− mice (Wagner et al., 2003) or C57BL/6 mice (Charles River). For RNAi experiments in primary macrophages, cells were transfected with control or smart-pool siRNAs (100 nM, Dharmacon) directed against NCoR, Ubc9, HDAC4, and TAB2 by using lipofectamine 2000 (Invitrogen). Cells were used for experiments after 48 hr incubation, and target gene knockdown was validated by QPCR (Figure S1). RAW 264.7 cells were transiently transfected with *inos*, *abca1*, or *mcp-1* promoters directing luciferase expression by using Superfect reagent (Qiagen). A β-galactosidase expression vector was cotrasfected as an internal control. For siRNA experiments in RAW264.7 macrophages, cells were transfected with siRNAs (40 nM) using Superfect reagent for 48 hr.

ChIP Assays

ChIP assays were performed as previously described (Pascual et al., 2005). Anti-LXR (Calbiochem), anti-PPARγ (ActiveMotif), and anti-NCoR (Affinity Bioreagents) antibodies were used. Rabbit preimmune serum was used as a control for nonspecific binding. 150 bp regions of the *inos*, *mcp-1*, *il1*β, and *tnf*α proximal promoters were amplified by real-time PCR. Primer sequences are available upon request.

RNA Isolation and QPCR

Total RNA (isolated by RNeasy kit, Qiagen) was prepared from primary macrophages. One microgram of total RNA was used for cDNA synthesis, and 1 μl of cDNA was used for real-time PCR with inflammatory-gene-specific primers. Primer sequences are available upon request. PCR (SYBERgreen) analysis was performed on an Applied Biosystems 7300 real-time PCR system. Values are normalized with GAPDH content.

CoIP Assay

HeLa cells were transfected with Flag-LXRβ and Ha-HDAC4 and lysed in low-salt lysis buffer. Ha-HDAC4 was immunoprecipitated with anti-HA beads (Covance) and Flag-LXRβ was detected with anti-Flag antibody (Sigma).

In Vitro SUMOylation Assay

In vitro SUMOylation reactions were carried out in 20 μl of reaction buffer by adding recombinant SUMO, SAE-1, SAE-2, Ubc9, and in vitro-translated HDAC4 or HDAC4 mutant (67-257) to the in vitro-translated LXRβ. Reactions were incubated for 60 min at 37°C supplemented with 2 mM ATP and analyzed by SDS-PAGE and immunoblotted with anti-HA (Covance) antibody.

In Vivo SUMOylation Assay

Whole-protein extract was prepared in the presence of NEM (N-Ethyl-maleimide, Calbiochem) from HeLa cells transfected with vectors directing expression of Flag-tagged wild-type LXRα and Flag-LXRβ, Myc-tagged SUMO1, SUMO2, or SUMO3, Ubc9, and wild-type HDAC4 or HDAC4 mutant (67-257) . Extracts were resolved by SDS-PAGE and immunoblotted with anti-Flag (Sigma) or anti-Myc (Santa Cruz) antibodies.

Supplemental Data

Supplemental Data include five figures and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/25/1/57/DC1/.

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Accession Numbers

Microarray data have been deposited in the Protein Data Bank under accession numbers GSE6346 and GSE6352. Please note that GSE6346 also contains data that were previously published in Ogawa et al. (2005).