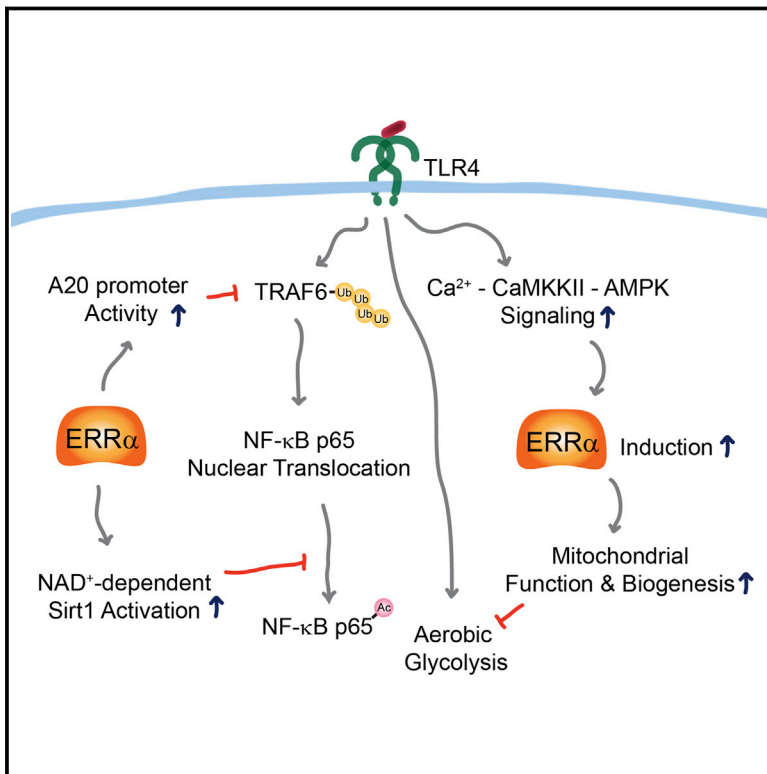# Orphan Nuclear Receptor ERRα Controls Macrophage Metabolic Signaling and A20 Expression to Negatively Regulate TLR-Induced Inflammation

Graphical Abstract

Authors

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In Brief

The orphan nuclear receptor ERRα is well known for its regulation of energy metabolism and mitochondrial biogenesis. Jo and colleagues show that ERRα also negatively regulates TLR4 signaling in macrophages through transcriptional activation of A20 and control of cellular metabolic reprogramming.

Highlights

- ERRα deficiency leads to excessive systemic and macrophage inflammatory responses

- ERRα negatively regulates TLR4 signaling through transcriptional activation of *A20*

- ERRα regulates metabolic reprogramming during TLR-induced inflammation

- Loss of ERRα promotes NF-κB signaling through p65 acetylation via SIRT1 inhibition

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Orphan Nuclear Receptor ERRα Controls Macrophage Metabolic Signaling and A20 Expression to Negatively Regulate TLR-Induced Inflammation

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SUMMARY

The orphan nuclear receptor estrogen-related receptor α (ERRα; NR3B1) is a key metabolic regulator, but its function in regulating inflammation remains largely unknown. Here, we demonstrate that ERRα negatively regulates Toll-like receptor (TLR)-induced inflammation by promoting *Tnfaip3* transcription and fine-tuning of metabolic reprogramming in macrophages. ERRα-deficient (*Esrra*^{−/−}) mice showed increased susceptibility to endotoxin-induced septic shock, leading to more severe pro-inflammatory responses than control mice. ERRα regulated macrophage inflammatory responses by directly binding the promoter region of *Tnfaip3*, a deubiquitinating enzyme in TLR signaling. In addition, *Esrra*^{−/−} macrophages showed an increased glycolysis, but impaired mitochondrial respiratory function and biogenesis. Further, ERRα was required for the regulation of NF-κB signaling by controlling p65 acetylation via maintenance of NAD^{+} levels and sirtuin 1 activation. These findings unravel a previously unappreciated role for ERRα as a negative regulator of TLR-induced inflammatory responses through inducing *Tnfaip3* transcription and controlling the metabolic reprogramming.

INTRODUCTION

Toll-like receptors (TLRs) are the most characterized innate immune receptors. They play a key role in the recognition of invading pathogens and the detection of various danger signals, triggering intracellular signaling cascades that initiate inflammatory and antimicrobial responses (Kawai and Akira, 2011). To avoid excessive inflammatory responses, TLR signaling pathways are tightly controlled by multiple levels of transcriptional and post-translational regulation (Kawai and Akira, 2011). Indeed, numerous negative regulators have been identified that regulate host innate immune signaling pathways, thereby preventing uncontrolled and potentially harmful inflammatory responses (Kondo et al., 2012). A crucial adaptor of TLR signaling, TNF receptor-associated factor (TRAF) 6, is targeted by multiple negative regulators, including A20/TNFAIP3, Cylindromatosis (CYLD), TANK, and small heterodimer partner (SHP) (Boone et al., 2004; Kawagoe et al., 2009; Trompouki et al., 2003; Xia et al., 2013; Yoshida et al., 2005; Yuk et al., 2011).

Estrogen-related receptor α (ERRα), ERRβ, and ERRγ (NR3B1, NR3B2, and NR3B3, respectively) are orphan nuclear receptors that share characteristics with, but are distinct from, ER-α (NR3A1) and ER-β (NR3A2) (Deblois and Giguère, 2013). ERRα was the first identified orphan nuclear receptor, with a crucial role in regulating energy metabolism and mitochondrial biogenesis (Deblois and Giguère, 2011; Villena and Kralli, 2008). ERRα, like other ERRs (ERRβ and ERRγ), are transcription factors that translate metabolic signals into specific gene-expression profiles, to fulfill the energetic demands required for diverse

biological functions (Giguère, 2008). Emerging evidence suggests that the ERR family members might have therapeutic potential for the treatment of metabolic diseases and cancers (Ariazi and Jordan, 2006; Deblois and Giguère, 2011, 2013). Previous studies have shown that ERRα contributes to interferon-γ-induced antimicrobial responses to *Listeria* infection via a mechanism involving expression of the mitochondrial respiratory chain machinery and increased mitochondrial reactive oxygen species (ROS) generation (Sonoda et al., 2007). In addition, ERRα is involved in the regulation of metabolism essential for effector T cell activation and differentiation (Michalek et al., 2011). Recent studies have indicated that ERRα promotes hepatocellular carcinoma by enhancing NF-κB activity and the inflammatory responses (Hong et al., 2013). Another recent study showed that an ERRγ inverse agonist exerts a protective effect against intracellular microbial infection (Kim et al., 2014). Although these reports provide insight into the potential role of the ERR family members in the immune response and inflammation, functional studies regarding the specific role of each ERR in the regulation of the immune and inflammatory responses are in their infancy. In the study described here, we aimed to characterize the role of ERRα in the regulation of TLR-induced inflammatory responses.

RESULTS

ERRα Deficiency Exacerbates Systemic Inflammatory Responses In Vivo, Leading to Increased Mortality

We first examined the role of ERRα in the regulation of systemic inflammation in vivo. ERRα protein was detected in the liver, spleen, and bone marrow (BM) in wild-type (WT) mice after intraperitoneal injection of lipopolysaccharide (LPS) at 8 hr (data not shown). To determine whether ERRα deficiency affects susceptibility to systemic inflammation, we challenged *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ mice with endotoxin-induced septic shock. As shown in Figure 1A, the lethality associated with septic shock was dramatically increased in *Esrra*$^{-/-}$ mice, when compared with that in *Esrra*$^{+/+}$ mice. Consistent with the diminished survival rates, the levels of pro-inflammatory cytokines increased significantly in *Esrra*$^{-/-}$ mice after LPS challenge (Figure 1B). Next, we assessed the production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and IL-1β in different tissues (lung, spleen, and liver) in response to LPS injection. The mRNA expression of *Tnf*, *Il6*, and *Il1b* in response to LPS challenge was greatly elevated in the lung and spleen tissues of *Esrra*$^{-/-}$ mice, compared with the corresponding tissues of *Esrra*$^{+/+}$ mice (Figure 1C). In addition, neutrophil infiltration was markedly elevated in the lung tissues of *Esrra*$^{-/-}$ mice, compared with *Esrra*$^{+/+}$ mice, after LPS challenge (Figure 1D). These results indicate that ERRα attenuates systemic inflammation and reduces lethality in septic shock models.

We next explored the contribution of BM–derived ERRα in endotoxin-induced septic shock and inflammation by using BM chimeric mice generated from *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ mice. The generation of ERRα BM chimeras was performed by the following adoptive transfer between histocompatible mouse strains: reconstitution of *Esrra*$^{-/-}$ mice with *Esrra*$^{+/+}$ or *Esrra*$^{-/-}$ BM-derived cells, and reconstitution of *Esrra*$^{+/+}$ mice with *Esrra*$^{-/-}$ or *Esrra*$^{+/+}$ BM-derived cells. Gene analysis of ERRα

showed the expression of ERRα in peripheral leukocytes from *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ mice transplanted with *Esrra*$^{+/+}$ mouse-derived BM cells (data not shown). When the mice were subjected to endotoxin-induced lethal shock, both *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ mice transplanted with *Esrra*$^{+/+}$ mouse-derived BM cells had lower expression of *Tnf* and *Il6* mRNA in spleen (Figure 1E), lower concentrations of TNF and IL-6 in serum (Figure 1F), and better survival (n = 10; Figure 1G) than in the respective *Esrra*$^{-/-}$ BM recipients. These data indicate that *Esrra*$^{+/+}$ BM-derived cells essentially contribute to regulating the sensitivity of animals to endotoxin-induced lethal shock.

ERRα Attenuates TLR4-Induced Inflammatory Response by Regulation of NF-κB Signaling in Macrophages

We next compared pro-inflammatory cytokine generation between *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ macrophages. As shown in Figures 2A and 2B, the mRNA and protein levels of TNF, IL-1β, and IL-6 were higher in LPS-stimulated BM-derived macrophages (BMDM) from *Esrra*$^{-/-}$ mice, when compared to those from *Esrra*$^{+/+}$ cells. These results were also confirmed by knockdown experiments using lentiviral short hairpin RNA (shRNA) specific for *Esrra* (shEsrra) (data not shown). Additionally, ERRα deficiency resulted in an enhancement of IKKα-IKKβ phosphorylation and IκBα degradation (Figures 2C and 2D), NF-κB reporter activity (Figure 2E), and nuclear translocation of p65 (Figure 2F), after LPS stimulation. The posttranslational modification of NF-κB (i.e., acetylation of NF-κB) is crucial to enhance the DNA-binding activity of NF-κB p65 and gene activation of pro-inflammatory mediators (Huang et al., 2010; Perkins, 2006). LPS stimulation resulted in increased acetylation of NF-κB p65 in *Esrra*$^{-/-}$ BMDM (Figure 2G), especially in the nucleus (Figures 2H and 2I), compared to control BMDM. Together, these results indicate that ERRα negatively regulates TLR4-induced NF-κB signaling activation in macrophages.

Furthermore, the expression of ERRα was strongly upregulated by stimulation with various TLR agonists, including Pam3CSK4 (a ligand for TLR1 and TLR2), zymosan (TLR2 and Dectin-1 ligand), and CpG-DNA (a TLR9 ligand) (Figure S1A). The production of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-12p40) in response to Pam3CSK4, zymosan, and CpG DNA was greatly increased in *Esrra*$^{-/-}$ cells compared with *Esrra*$^{+/+}$ cells (Figure S1B). However, TLR3-induced expression of *Tnf* mRNA was not affected by ERRα deficiency (data not shown). Thus, ERRα regulates the MyD88-dependent signaling pathway, but not the TRIF-dependent signaling pathway.

ERRα Enhances *Tnfaip3* Promoter Activity, but Inhibits TRAF6 Ubiquitination, in Response to LPS

We next investigated the molecular mechanisms by which ERRα negatively regulates TLR4-signaling pathways. To address this issue, we performed global gene-expression analysis of *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ BMDM after LPS stimulation. Microarray analysis and quantitative real-time PCR (qRT-PCR) analysis revealed that several genes encoding various TLR-negative regulators including *Tnfaip3* (Boone et al., 2004), *Socs1* (Mansell et al., 2006), *Tank* (Kawagoe et al., 2009), *Senp6* (Liu et al., 2013), and *Rnf216* (Chuang and Ulevitch, 2004) were downregulated in LPS-stimulated *Esrra*$^{-/-}$ BMDM, when compared with *Esrra*$^{+/+}$ BMDM (Figures 3A and 3B). Moreover, these gene sets, which
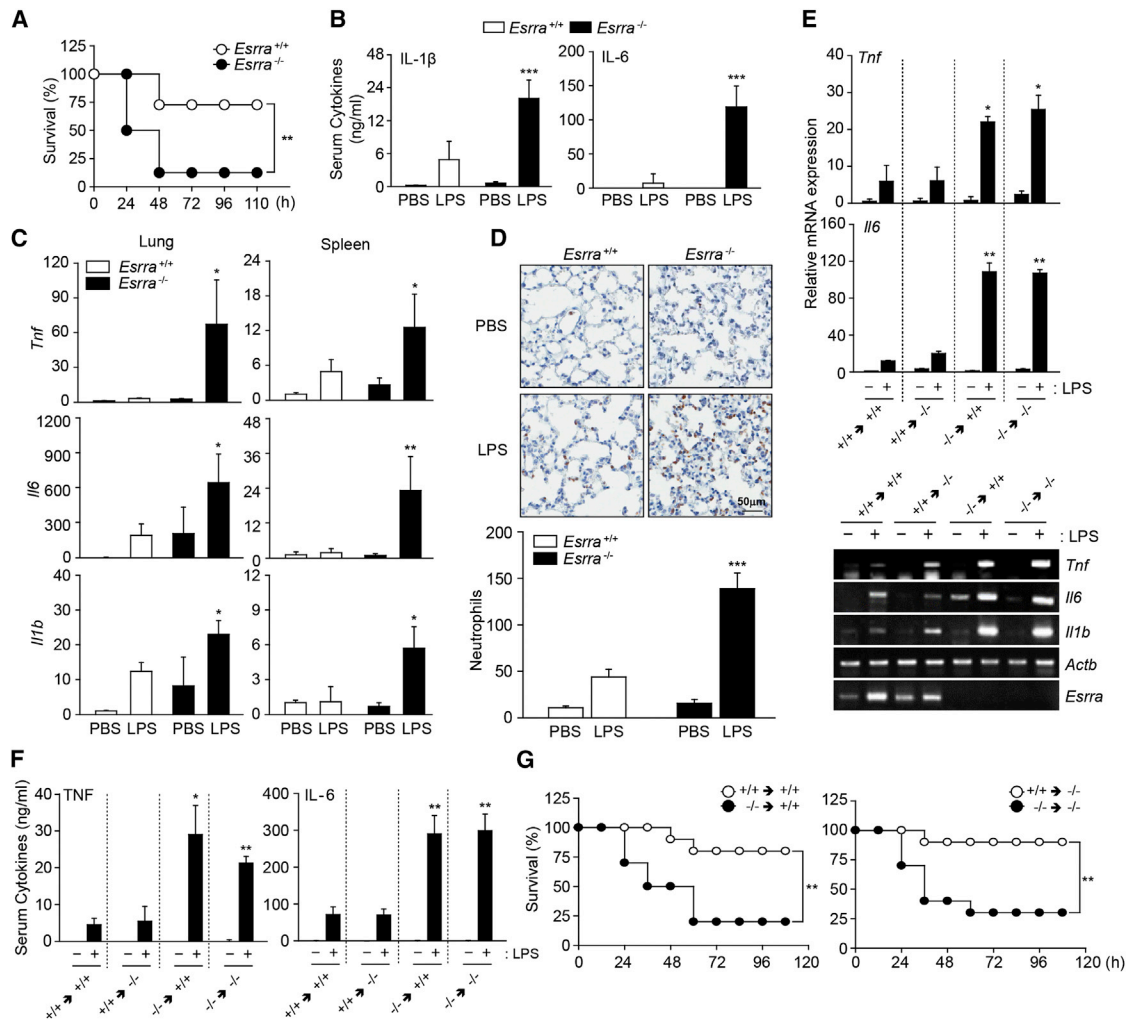
Figure 1. ERRα Is Required for Protection from LPS-Induced Lethal Shock

(A–D) *Esrra*^{+/+} and *Esrra*^{−/−} mice challenged with LPS (20 mg/kg, i.p.). Data are presented as mean ± SD. (A) Survival curves (n = 11). (B) Serum concentrations of IL-1β and IL-6. (C) qPCR analysis of *Tnf*, *Il6*, and *Il1b* mRNA expression in lung (left) and spleen (right) samples at 18 hr post-LPS injection (n = 3). (D) Neutrophil counts in lung tissues (n = 3). Top, representative images (scale bar represents 50 μm); bottom, numbers of infiltrating neutrophils counted from 10 random fields. (E–G) ERRα BM-chimeric mice (10 weeks old) injected with LPS (20 mg/kg, i.p.). Data are presented as mean ± SD. (E) qPCR (top) or semiquantitative (down; representative images) RT-PCR analysis of *Tnf* and *Il6* mRNA expression from spleen tissues. (F) Serum concentrations of TNF-α and IL-6 at 18 hr post-LPS injection (n = 3). (G) Survival curves (n = 10).
*p < 0.05, **p < 0.01, ***p < 0.001, compared with control mice stimulated with LPS (log-rank test [A and G] or two-tailed Student's t test [B–F]).

are involved in the negative regulation of TLR signaling, were up-regulated by ERRα overexpression (Figure S2A).

Consistent with the microarray data, mRNA and protein expression of A20 (Figures 3C and 3D) were significantly attenuated in LPS-stimulated *Esrra*^{−/−} macrophages, when compared with *Esrra*^{+/+} macrophages. ERRα binds to the *Esrra* consensus motif (5′-TNAAGGTCA-3′) in order to regulate transcriptional activity of target genes (Sladek et al., 1997). We further determined whether the ERRα plays a role in the transcriptional activation of *Tnfaip3*. Silencing and overexpression of ERRα respectively decreased (Figure 3E) and increased (Figure 3F) *Tnfaip3* promoter activity in RAW264.7 cells after LPS stimulation. In addition, transfection with the mutants deleting the *Esrra* consensus motif in the *Tnfaip3* promoter showed a marked inhibition of reporter gene activity (Figures 3E and 3F).

Since the *Tnfaip3* gene contains the *Esrra* consensus motif (−520/−513) in its promoter region, we evaluated the recruitment of ERRα to the *Tnfaip3* promoter in macrophages. After LPS stimulation, ERRα binding to the *Tnfaip3* promoter region increased significantly, whereas ERRα binding activity to the irrelevant promoter sites (−1740/−1586) decreased markedly in macrophages (Figure 3G). Analysis of the ERRα chromatin immunoprecipitation (ChIP)-seq data from mouse liver in our previous work (Chaveroux et al., 2013) revealed over 20 ERRα-bound genes involved in TLR signaling (Figure S2B, top). ChIP analysis and qPCR in BMDM show ERRα recruitment to *Tank*, *Senp6*, and *Rnf216* (*Triad3*), several known negative regulators of TLR signaling (Figure S2B, bottom). Because A20 is a well-known deubiquitinase in TLR signaling (Boone et al., 2004), we examined whether ERRα deficiency affected TLR4-induced
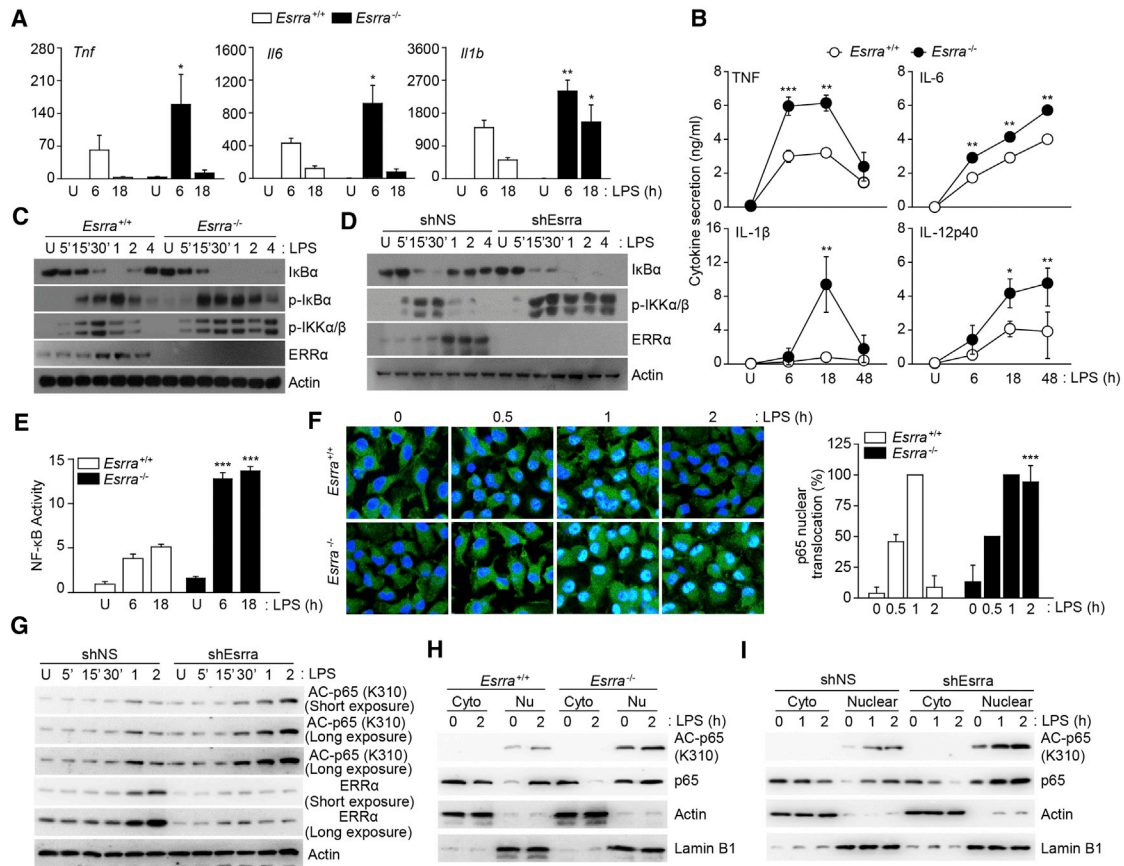
Figure 2. ERRα Deficiency Excessively Activates TLR4-Dependent Inflammatory Responses through Enhanced Activation of NF-κB Signaling

(A–C, E, F, H) *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ BMDM were incubated with LPS (100 ng/mL) for the indicated times.

(A) qPCR analysis of *Tnf*, *Il6*, *Il1b*, and *Il12p40* mRNA expression.

(B) ELISA analysis of TNF-α, IL-6, and IL-1β levels in culture supernatants.

(C) Immunoblot analysis of the indicated phosphorylated (P-) and total proteins.

(D, G, I) BMDM were transduced with lentiviruses expressing nonspecific shRNA (shNS) or shRNA specific for *Esrra* (shEsrra), followed by LPS stimulation for the indicated time periods.

(D) Immunoblot analysis of the indicated phosphorylated (P-) and total proteins.

(E) Cells were transduced with adenovirus carrying NF-κB luciferase reporter constructs before LPS stimulation. Luciferase assays were performed based on normalization to the β-galactosidase activity.

(F) Immunofluorescence microscopy analysis of NF-κB p65 nuclear translocation. Left, representative images; right, quantitation of p65 nuclear translocation.

(G) Immunoblot analysis of the indicated acetylated (AC-) and total proteins.

(H and I) Immunoblot analysis of the indicated acetylated (AC-) and total proteins in the cytoplasmic (Cyto) and nuclear (Nu) extracts. Lamin B1 and β-actin are used as quality and loading controls.

Data are representative of three independent experiments and are presented as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, compared with control cultures (two-tailed Student's t test). Also see Figure S1.

TRAF6 polyubiquitination. We found that K63-linked polyubiquitination of TRAF6 increased, whereas K48-linked polyubiquitination decreased, in *Esrra*$^{-/-}$ BMDM after LPS stimulation (Figures 3H–3J). Together, ERRα inhibits TLR signaling via transcriptional activation of the *Tnfaip3* gene, thus inhibiting K63-linked polyubiquitination of TRAF6.

ERRα Regulates TLR4-Mediated Glycolytic Metabolism, NF-κB p65 Acetylation, and HIF1α Stabilization

On the basis of the findings that ERRα is required for mitochondrial biogenesis and function of OXPHOS (Hong et al., 2013; Sonoda et al., 2007), we assessed glycolysis and the NAD$^+$/ NADH ratio in macrophages. When compared to *Esrra*$^{+/+}$ BMDM, *Esrra*$^{-/-}$ BMDM showed a marked inhibition of the NAD$^+$/NADH ratio from 91.8% to 51.3% (at 8 and 18 hr, respectively) (Figure 4A), an increase of lactate levels (Figure 4B) after LPS stimulation. In addition, the extracellular acidification rate (ECAR), an indirect indicator of lactate production and enhanced glycolytic metabolism (Doherty and Cleveland, 2013), levels were significantly elevated in *Esrra*$^{-/-}$ BMDM at 8 hr (Figure 4C, top) and 18 hr (Figure 4C, bottom) of LPS stimulation. However, there was no significant difference between *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ cells in LPS-induced ATP production (Figure 4D). To clearly establish the origin of ATP generation, we replaced glucose
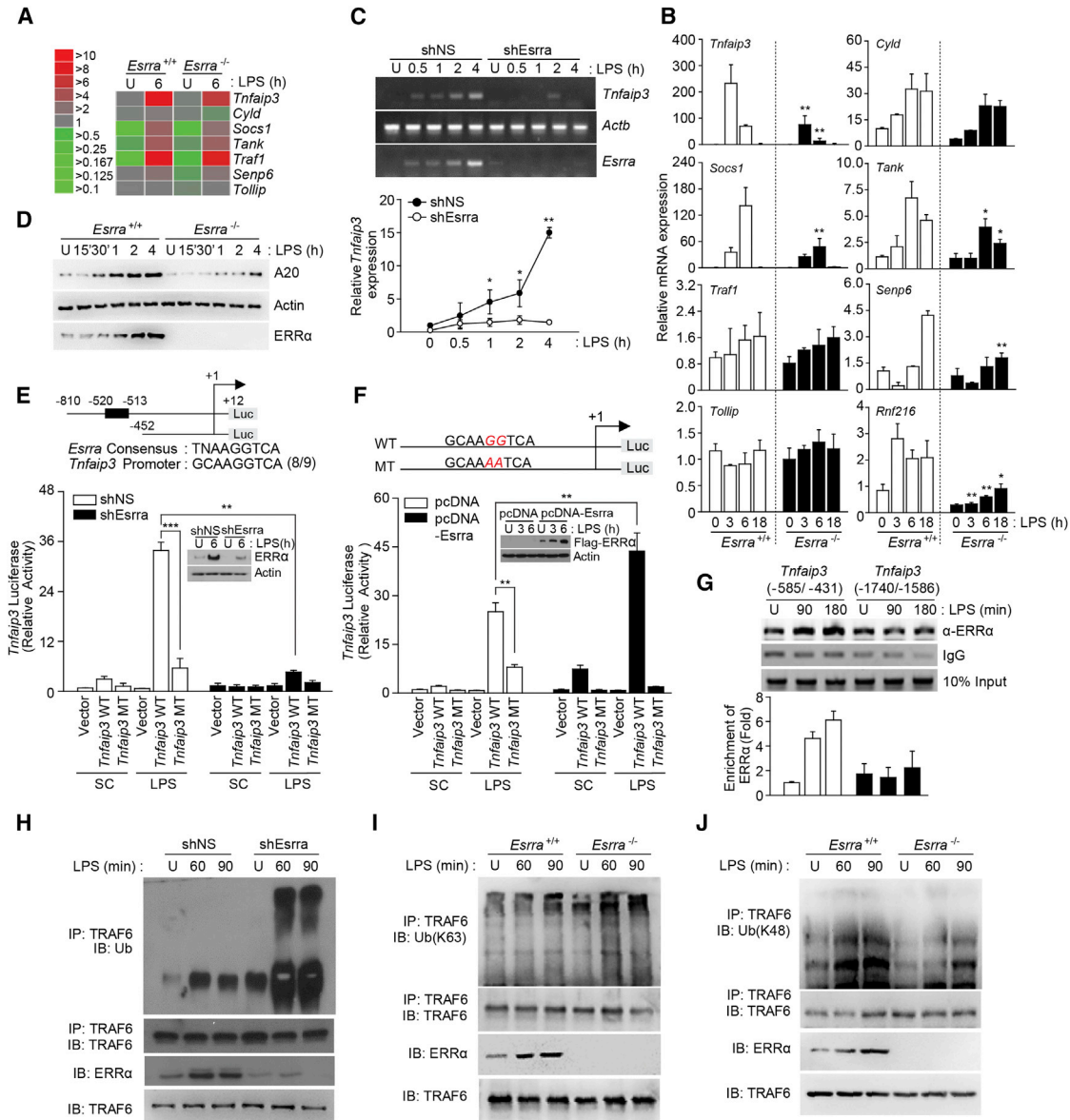
Figure 3. ERRα Promotes A20 Transcriptional Activation, and Inhibits TRAF6 Ubiquitination

(A, B, D, I, and J) *Esrra*^{+/+} and *Esrra*^{−/−} BMDM were incubated with LPS (100 ng/mL) for the indicated times.

(A) Heat map representing LPS-responsive gene-expression profiles including TLR-negative regulators in *Esrra*^{+/+} and *Esrra*^{−/−} BMDM. Each column in the heat map represents the ratio of normalized expression.

(B) qPCR analysis of *Tnfaip3*, *Socs1*, *Traf1*, *Tollip*, *Cyld*, *Tank*, *Senp6*, and *Rnf216* mRNA expression.

(C and H) BMDM were transduced with shNS or shEsrra, followed by LPS stimulation for the indicated time periods. Semiquantitative RT-PCR (top) or qPCR (bottom) analysis for *Tnfaip3* expression. β-actin served as loading controls.

(D) Immunoblot analysis of the indicated proteins.

(E and F) RAW264.7 cells were transduced/transfected before LPS stimulation (6 hr). Luciferase assays were performed based on normalization to the β-galactosidase activity. Top shows diagrams that show deletions (E) or point mutations (F) of ERRα-binding sites in the *Tnfaip3* promoter constructs used for the luciferase assay.

(E) RAW264.7 cells transduced with shNS or shEsrra were transfected with a *Tnfaip3*-luciferase reporter construct carrying wild-type (WT) or deleted (MT) ERRα-binding site. Inset shows knockdown efficiency of ERRα protein.

(F) RAW264.7 cells were transfected with a *Tnfaip3*-luciferase reporter construct (WT, *Tnfaip3* WT; MT, *Tnfaip3* carrying point mutations in the ERRα-binding site) in the presence or absence of the plasmid encoding flag-tagged murine ERRα (pcDNA-Esrra). Inset shows transfection efficiency of ERRα plasmid.

(G) ChIP assays were conducted in BMDM stimulated with LPS (90 or 180 min). Immunoprecipitations were performed using Abs specific to ERRα, and conventional RT-PCR (top) or qPCR analysis (bottom) using primers specific to the *Tnfaip3* promoter.

(H) Immunoprecipiation of TRAF6 from LPS-stimulated control BMDM (shNS), and BMDM in which ERRα was knocked down (shEsrra), followed by immunoblot analysis with anti-ubiquitin (Ub) or anti-TRAF6. Bottom shows immunoblot analysis of ERRα or TRAF6 in lysates without precipitation.

(legend continued on next page)

with galactose in LPS-treated cells. LPS-induced ATP generation was effectively blocked by galactose in *Esrra*$^{-/-}$ cells, supporting the contention that ATP generation in this context is glycolysis-dependent (Figure 4E). Consistent with this, ChIP-qPCR data showed that several genes involved in glycolysis including *Pdk4* (an inhibitor of the conversion of pyruvate to acetyl-CoA) and *Slc2a1* (glucose transporter 1), was elevated in *Esrra*$^{-/-}$ BMDM compared with WT BMDM (Figure S2B, bottom).

Sirtuin 1 (SIRT1) is an NAD$^+$-dependent deacetylase that is critically involved in the regulation of NF-κB-mediated inflammatory responses via the deacetylation of p65/RelA on lysine 310 (Chen et al., 2005; Yang et al., 2012; Yang et al., 2006; Yeung et al., 2004). We evaluated the influence of ERRα on SIRT1-mediated deacetylation of NF-κB p65. Following LPS stimulation, cellular NAD$^+$ levels (Figure S3A) and SIRT1 mRNA and protein expression were decreased in *Esrra*$^{-/-}$ macrophages compared to those from control macrophages (Figures 4F, 4G, and S3B). Simultaneously, ERRα deficiency in macrophages led to enhanced acetylation of NF-κB p65 on Lys310 after LPS stimulation (Figures 4F and S3B). In addition, ERRα overexpression resulted in *Sirt1* promoter induction, increasing the reporter gene activities 2.4-fold (Figure 4H). However, a mutation within the *Esrra* consensus motif of the *Sirt1* promoter (Figure 4H, top) decreased the response to LPS in RAW264.7 cells (Figure 4H). Taken together, these findings suggest that the *Sirt1* promoter is responsive to ERRα and that the identified ERR response element (ERRE) contributes to the induction by ERRα.

Previous studies have demonstrated that HIF1α is an important activator of aerobic glycolysis that is stabilized by the LPS-induced metabolite succinate (Tannahill et al., 2013). Further investigation of LPS-induced protein expression of HIF1α confirmed that HIF1α is upregulated and stabilized in *Esrra*$^{-/-}$ macrophages (Figure 4I). Silencing of *Hif1α* in macrophages indicated that LPS-induced HIF1α expression is essential for IL-1β production after LPS stimulation (Figure 4J), a result that is partially consistent with data from previous studies (Tannahill et al., 2013). Together, these results indicate that ERRα is required for controlling glycolysis, SIRT1-dependent NF-κB p65 acetylation, and HIF1α stabilization, all of which lead to enhanced inflammatory responses in macrophages in response to TLR4 stimulation.

ERRα Modulates Expression of Genes Regulating OXPHOS and Mitochondrial Biogenesis in Response to LPS

To evaluate whether ERRα deficiency affects mitochondrial function and biogenesis in macrophages, we next performed an extracellular flux analysis. LPS stimulation led to a characteristic decline in the oxygen consumption rate (OCR) in WT BMDM (Figure 5A), as previously shown (Tannahill et al., 2013). Compared to *Esrra*$^{+/+}$ BMDM, *Esrra*$^{-/-}$ BMDM exhibited a greater reduction in baseline OCR and carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP)-induced uncoupling during OXPHOS, both in

the presence and absence of LPS (Figure 5A). In addition, LPS treatment of *Esrra*$^{+/+}$ BMDM upregulated the protein expression of mitochondrial respiratory chain complexes I (NDUFA9), IV (COX4), and V (ATP5A1) (Figure 5B). Notably, the expression of most proteins of the respiratory chain complexes I–V was significantly diminished in *Esrra*$^{-/-}$ BMDM, at rest and at each time point after LPS stimulation compared to those in *Esrra*$^{+/+}$ BMDM (Figure 5B).

The microarray expression data showed that ERRα contributes to the macrophage induction of genes involved in mitochondrial biogenesis and metabolic functions (e.g., mitochondrial respiration, OXPHOS, ATP synthesis, tricarboxylic acid (TCA) cycle, and fatty acid β-oxidation; Figure S4). We further examined the gene and protein expression related to mitochondrial biogenesis in *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ BMDM before and after LPS stimulation. The expression of both nuclear respiratory factor (NRF)-1 and TFAM was affected by the absence of ERRα. In addition, after LPS stimulation, the genes and proteins involved in mitochondrial biogenesis, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), PGC1β, NRF-1, and TFAM, were significantly downregulated in *Esrra*$^{-/-}$ BMDM compared to *Esrra*$^{+/+}$ BMDM (Figures 5C and 5D). Moreover, LPS-induced mitochondrial DNA content (Figure 5E) and mass (Figure 5F) substantially decreased in *Esrra*$^{-/-}$ BMDM compared to *Esrra*$^{+/+}$ BMDM, suggesting that ERRα is required for enhancement of mitochondrial biogenesis (Figures 5E and 5F). Together, these data indicate that ERRα contributes to mitochondrial biogenesis, OXPHOS expression, and function in macrophages during TLR4 signaling.

TLR4 Activates Induction of ERRα via the Ca^{2+}-CaMKKβ-AMPK Pathway and MyD88 Signaling

Because TLR signaling induces expression of ERRα mRNA and protein (Figures 3C, 6A, and S1A), we next examined the molecular mechanism occurs in macrophages. Previous studies reported a connection between AMPK and ERRα in cardiac myocytes (Hu et al., 2011). Indeed, presentation of AMPK inhibitor (compound C) to macrophages markedly inhibited LPS-induced ERRα expression (Figure 6B). Overexpression of a constitutively active AMPK upregulated (Figures 6C and S5A), whereas transfection of dominant negative AMPK or knocking down of *Prkaa1* effectively attenuated, LPS-induced ERRα mRNA and protein expression in macrophages (Figures 6C and 6E; Figures S5A and S5B). Further, pharmacological inhibition or silencing of Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ), an upstream kinase of AMPK (Hardie, 2007), significantly attenuated ERRα expression in macrophages (Figures 6D, 6E, and S5B). However, blockade of LKB1, which also activates AMPK in macrophages (Hardie, 2007), did not inhibit LPS-induced ERRα expression (Figures 6F and S5C). Moreover, pharmacological inhibition of intracellular calcium release blocked LPS-induced ERRα expression and AMPK activation in macrophages (Figures S5D–S5F).
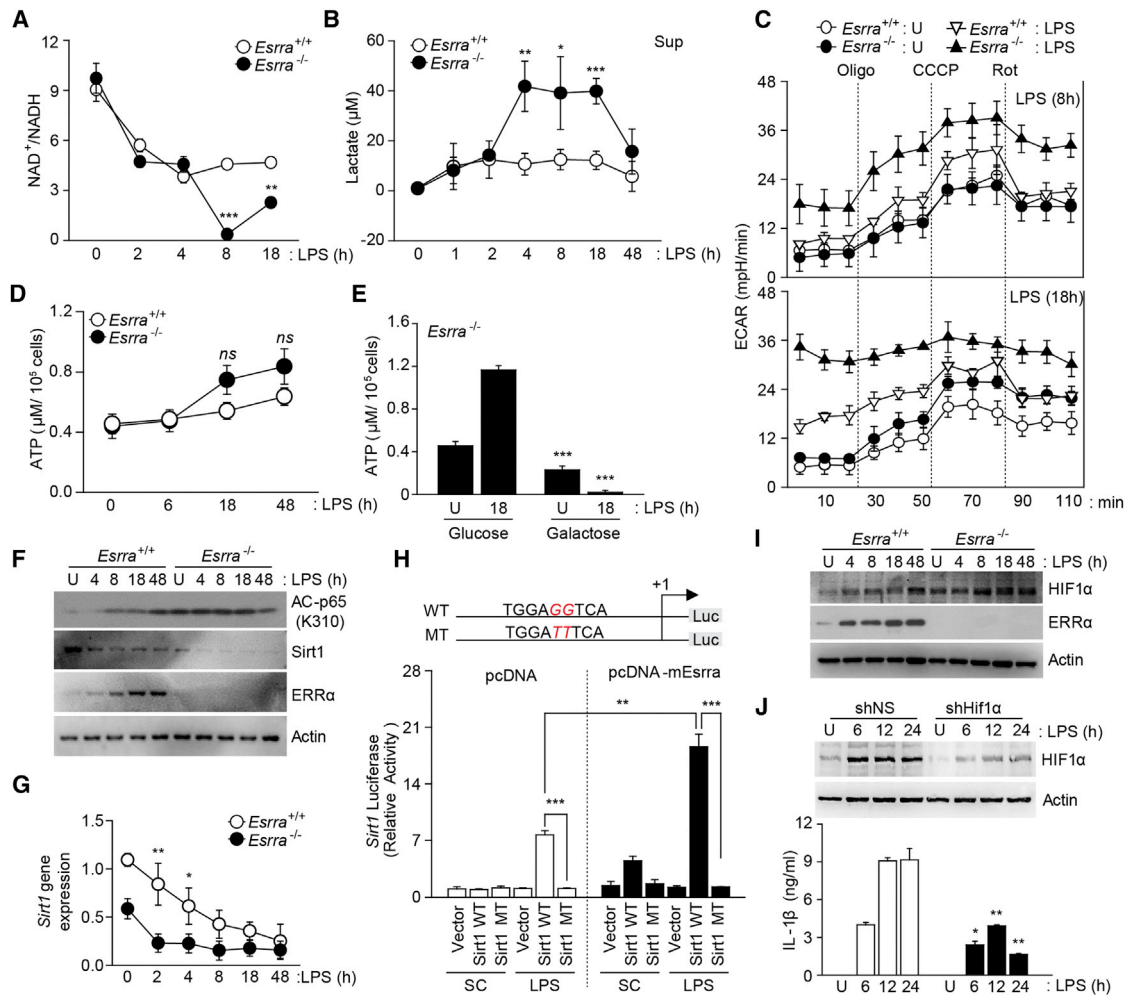
Figure 4. *Esrra*$^{-/-}$ Macrophages Enhances Aerobic Glycolysis, Hyperacetylation of NF-κB p65, and HIF1α Stabilization after LPS Stimulation

(A–D, F, G, I) *Esrra*$^{+/+}$ and *Esrra*$^{-/-}$ BMDM were incubated with LPS (100 ng/mL) for the indicated times.

(A) NAD$^+$/NADH ratio.

(B) Lactate production.

(C) The real-time rates of extracellular acidification rate (ECAR) were analyzed as an indicator of glucose oxidation. LPS stimulation for 8 hr (top) or 18 hr (bottom).

(D) Total ATP levels measured by the luciferin/luciferase method.

(E) Total ATP levels from *Esrra*$^{-/-}$ BMDM stimulated with LPS (100 ng/mL) for 18 hr, prior to culturing in glucose- or galactose-containing medium for 15 min.

(F) Immunoblot analysis of the indicated acetylated (AC-) and total proteins.

(G) qPCR analysis for *Sirt1* mRNA expression.

(H) RAW264.7 cells were transfected with a *Sirt1*-luciferase reporter construct (WT, *Sirt1* WT; MT, *Sirt1* carrying point mutations in the ERRα-binding site) in the presence or absence of the plasmid encoding flag-tagged murine ERRα (pcDNA-mEsrra). Cells were then stimulated with LPS (6 hr), followed by luciferase assays based on normalization to the β-galactosidase activity. Top shows diagrams that show point mutations (E) of ERRα-binding sites in the *Sirt1* promoter constructs used for the luciferase assay.

(I) Immunoblot analysis of the indicated proteins.

(J) BMDM were transduced with shNS or shRNA specific for *Hif1a* (shHif1a), followed by LPS stimulation for the indicated time periods. Top shows knockdown efficiency of HIF1α protein; Bottom shows ELISA analysis for IL-1β production.

Data are representative of three independent experiments and are presented as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, compared with control cultures (two-tailed Student's t test). Also see Figure S3.

PGC1 family members are critical for transcriptional control of mitochondrial biogenesis, and their activity is linked to the energy sensor AMPK (Scarpulla, 2011). We detected that AMPK activation was required for the induction of PGC1α (Figure S5G), which in turn promoted ERRα expression in response to LPS (Figures 6G and S5H). Indeed, PGC1α overexpression increased LPS-induced, ERRα-dependent transcriptional activity, in macrophages (Figure 6H). These data confirmed that the previously identified requirement of PGC1α for autoregulation of ERRα (Laganière et al., 2004) is also valid in macrophages. MyD88 and TRIF are important adaptors of TLR4-triggered activation of NF-κB signaling in the innate immune response (Kawai et al., 1999). LPS-induced expression of ERRα was partially abrogated in MyD88-deficient cells (Figure 6I), but not in
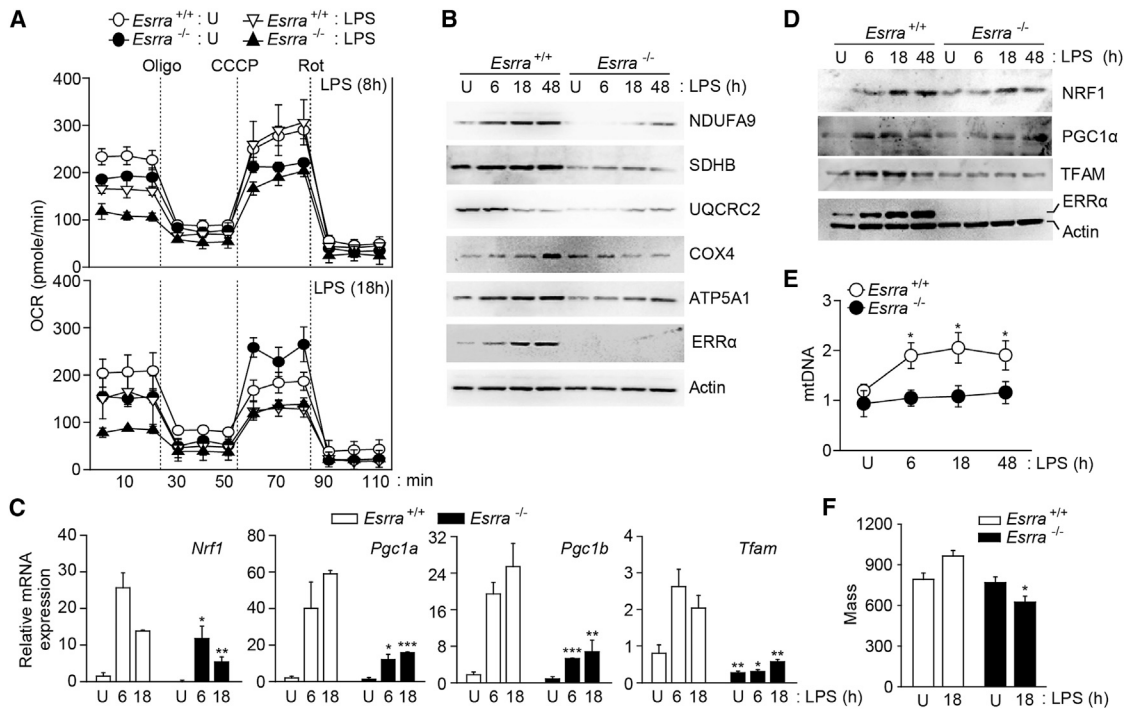
Figure 5. ERRα Deficiency Impairs Mitochondrial Respiration and Biogenesis during TLR4 Signaling

(A–F) Esrra^{+/+} and Esrra^{−/−} BMDM were incubated with LPS (100 ng/mL) for the indicated times.

(A) Real-time measurement of oxygen consumption rate (OCR) was analyzed by sequential treatment with oligomycin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and rotenone, as an indicator of oxidative metabolism.

(B) Immunoblot analysis of the indicated proteins.

(C) qPCR analysis for Nrf1, Ppargc1a (Pgc1a), Ppargc1b (Pgc1b), and Tfam expression.

(D) Immunoblot analysis of the indicated proteins.

(E) The mtDNA content was measured by qPCR analysis, and normalized by a nuclear DNA content.

(F) Measurements of mitochondrial mass using MitoTracker green with flow cytometry.

Data are representative of three independent experiments and are presented as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, compared with control cultures (two-tailed Student's t test). Also see Figure S4.

TRIF-deficient cells (Figure S5I). These data suggest that the Ca^{2+}-CaMKKβ-AMPK-PGC1α and TLR4-MyD88 signaling pathways are required for LPS-induced induction of endogenous ERRα in macrophages.

DISCUSSION

Here we demonstrated that ERRα regulates TLR signaling, via at least two mechanisms: dissociation of adaptor complexes through A20-mediated regulation of TRAF6 polyubiquitination and posttranslational regulation of NF-κB p65 acetylation, which was modulated by SIRT1 activation. We further showed that ERRα is involved in promoting mitochondrial respiratory function and biogenesis required to drive the more energy-efficient oxidative metabolic pathways during TLR-induced inflammation. Notably, BM-derived ERRα was required for controlling inflammatory responses during endotoxin stimulation in vivo. It is noteworthy that ERRα associates with distinct chromatin binding sites in the promoter region of Tnfaip3, and that ERRα is required for Tnfaip3 promoter activity of genes containing ERRα-binding elements. A20 deubiquitinates the signaling molecule TRAF6, thus disrupting ubiquitin-conjugated enzyme complexes and terminating TLR-induced NF-κB signaling and inflammatory re-

sponses (Boone et al., 2004; Shembade et al., 2010). Previous studies showed that ERRα null livers display increased NF-κB-dependent cytokine gene expression in the Kupffer cells, an effect associated with the disruption of ERRα-dependent regulation of the NF-κB inhibitor IκBα (Hong et al., 2013). Combined with the current findings, ERRα may modulate IκBα and other negative regulators (e.g., Tank, Senp6, Rnf216; see Figures 3B and S2) of the TLR-induced inflammatory response in macrophages (proposed mechanisms, Figure S6).

During inflammation, the early phase responses primarily require glycolysis, whereas fatty acid oxidation via NAD$^+$-dependent processes plays a more dominant role in later phases (Everts et al., 2014; Liu et al., 2012b; Liu et al., 2011). This study elucidated another key role of ERRα in the molecular checkpoint of the immune cell metabolism through the regulation of NAD$^+$-dependent processes, thus affecting inflammatory responses in immune cells. Emerging evidence indicates that the SIRT family of NAD$^+$ sensors is critically involved in linking metabolism and inflammation (Liu et al., 2012a; Liu et al., 2011; Yeung et al., 2004). The increased NAD$^+$ levels brought about by the Nampt salvage pathway in mammalian cells activate SIRT1 to deacetylate and inactivate NF-κB p65, thereby terminating inflammatory responses (Liu et al., 2011; Yeung et al., 2004). Aside from the
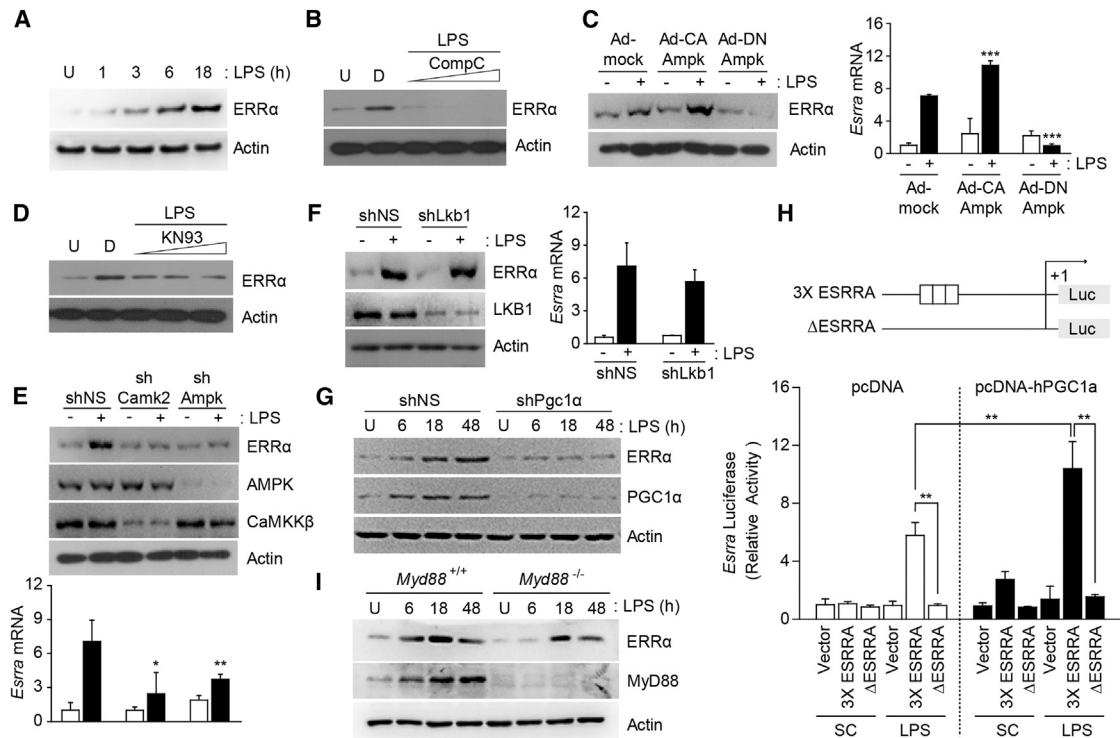
Figure 6. TLR4 Activation Induces Macrophage ERRα via the Ca²⁺-CaMKKβ-AMPK and TLR4-MyD88 Signaling

(A) Immunoblot analysis of ERRα in BMDM after LPS (100 ng/mL) stimulation for the indicated times.

(B and D) Immunoblot analysis of ERRα in BMDM after LPS stimulation (100 ng/mL; 6 hr) in the presence or absence of the AMPK inhibitor compound C (Comp C; 5, 10, 25 μM) (B) or the Ca²⁺/CaMKII inhibitor KN93 (5, 10, 25 μM) (D).

(C, E–G) BMDM were transduced with Ad-GFP, Ad-CA-AMPK, or Ad-DN-AMPK (C); shNS, shRNA specific for *Camk2* (shCamk2), or for *Ampka* (shAmpk) (E); transduced with shNS or shRNA specific for *Lkb1* (shLkb1) (F); shNS or shRNA specific for *Ppargc1a* (shPgc1a) (G) prior to stimulation with LPS (100 ng/mL) for 6 hr. (C Left, E Top, F Left, and G) Immunoblot analysis of the indicated proteins; (C Right, E Bottom, F Right) qPCR analysis of *Esrra*.

(H) THP-1 cells were transfected with *Esrra*-luciferase reporter constructs containing 3 copies of the *Esrra* element (3X) or a synthetic mutant in which the region containing the *Esrra* element is absent (ΔESRRA), in the presence or absence of the plasmid encoding flag-tagged human *PPARGC1A* (pcDNA-hPGC1a). Cells were then treated with LPS for 6 hr, followed by luciferase assays, which were normalized to β-galactosidase activity. Top shows diagrams that show *Esrra*-luciferase reporter constructs used for the luciferase assay.

(I) Immunoblot analysis of ERRα in *Myd88*⁺/⁺ and *Myd88*⁻/⁻ BMDM after LPS (100 ng/mL) stimulation for the indicated times.

Data are representative of three independent experiments and are presented as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, compared with control cultures (two-tailed Student's t test). Also see Figure S5.

findings that ERRα is required for preventing NAD⁺ depletion, we unexpectedly found that ERRα promotes the transcriptional activation of *Sirt1* during inflammation. Additionally, we showed that HIF1α stabilization is elevated in *Esrra*⁻/⁻ macrophages. These findings might be due to SIRT1 inactivation in ERRα deficiency, based on the previous findings that SIRT1 represses HIF1α-target genes through binding and deacetylating HIF1α (Lim et al., 2010). Further, we confirmed the central role of HIF1α in LPS-induced IL-1β expression. In accordance with this, previous findings reported that HIF1α stabilization leads to increased IL-1β secretion by macrophages (Tannahill et al., 2013).

In response to pathogenic or dangerous stimuli, immune cells undergo metabolic reprogramming, which shapes the innate immune responses to invading pathogens or tissue damage (Ganeshan and Chawla, 2014; Martinez et al., 2013). Indeed, mitochondrial biogenesis in response to inflammation is beneficial to the host in terms of preserving metabolic function and cell viability, as well as to assist with resolution of inflammation (Piantadosi and Suliman, 2012). Data presented here reveal that ERRα

contributes to mitochondrial biogenesis and OXPHOS function in TLR-activated macrophages through the activation of genes involved in these processes. These data are in partial agreement with previous studies showing that ERRα is essentially involved in the expression of numerous genes related to mitochondrial respiratory function (Sonoda et al., 2007). The same study also showed that IFN-γ-induced ERRα activation is mediated through PGC1β (Sonoda et al., 2007). In the adaptation phase of inflammatory responses, PGC1α and -β promote the expression of multiple genes including NRF-1 and NRF-2, which play roles in mitochondrial biogenesis and OXPHOS (Gerhart-Hines et al., 2007; Vercauteren et al., 2008). A previous study demonstrated that LPS stimulation leads to NRF-1 gene induction through co-regulation of NF-κB and CREB signaling, leading to attenuation of inflammatory damage by linking mitochondrial biogenesis (Suliman et al., 2010). Together, these data indicate that ERRα-dependent activation of genes involved in mitochondrial biogenesis and respiratory function could contribute toward ameliorating mitochondrial damage and

maintaining mitochondrial homeostasis, thus inhibiting excessive inflammatory responses during TLR signaling.

TLR signaling in macrophages leads to an initial reduction, followed by a delayed elevation of AMPK activation (Yuk et al., 2011). Increased AMPK activation enhances SIRT1 activity by increasing cellular NAD^+ levels, resulting in activation of PGC1 to promote mitochondrial biogenesis and induction of the metabolic shift to fatty acid oxidation (Cantó and Auwerx, 2009; Lin, 2009; Scarpulla, 2011). The PGC1α regulatory network is indicative of a critical role for ERRα in orchestrating mitochondrial biogenesis and energy homeostasis in many tissues, to serve diverse biological functions (Scarpulla, 2011). Notably, we found that LPS stimulation induced intracellular Ca^{2+}-dependent AMPKα phosphorylation, which is required for the expression of PGC1α, and that silencing of PGC1α diminished the TLR-induced increase in ERRα gene expression. The current study provides evidence for a new function of AMPK signaling as a negative feedback mechanism during inflammation through induction of the PGC1α/ERRα pathway (see Figure S6), thus driving mitochondrial biogenesis according to the metabolic requirements during inflammatory stresses.

In summary, our results suggest that ERRα is a novel regulator of the TLR-induced inflammatory response, with the unique capacity to modulate (1) *Tnfaip3* transcriptional induction and (2) p65 acetylation through metabolic reprogramming via enhancement of mitochondrial function. Through these regulatory processes, ERRα might control the overall inflammatory response to prevent excessive damage to the host. These findings provide a potential new therapeutic strategy for the treatment of systemic inflammatory response syndrome.

EXPERIMENTAL PROCEDURES

For mouse model of sepsis and bone marrow transplantation; reagents, antibodies, primer and plasmid construction; adenovirus production; lentiviral shRNA generation and transduction of primary cells; measurement of ATP production; immunoprecipitation and in vivo ubiquitination assays; and ChIP assay, luciferase assay, fluorescence microscopy, histology, and immunohistochemistry, see Supplemental Experimental Procedures.

Cell Preparation

BMDM were differentiated for 5–7 days in medium containing macrophage colony-stimulating factor, as described previously (Yuk et al., 2011). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin, and 5×10^{-5} M 2-mercaptoethanol. The mouse macrophage cell line RAW264.7 and HEK293 cells were purchased from the American Type Culture Collection and grown in DMEM/glutaMAX (Life Technologies) supplemented with 10% FBS.

RNA Extraction, qPCR, Semiquantitative RT-PCR, Western Blotting, Subcellular Fractionation, and Enzyme-Linked Immunosorbent Assays

RNA extraction, real-time quantitative PCR, and semiquantitative RT-PCR were performed as described previously (Yuk et al., 2011). The sequences of the primers used are described in the Supplemental Experimental Procedures. For Western blotting, the primary antibodies were used at a 1:1000 dilution. The membranes were developed using a chemiluminescence assay (ECL; Pharmacia-Amersham) and were subsequently exposed to chemiluminescence film (Pharmacia-Amersham). Nuclear and cytosolic protein extracts were prepared using the Nuclear Extraction Kit (Active Motif) according to

the manufacturer's instructions. In the sandwich ELISA, serum and cell culture supernatants were analyzed using DuoSet antibody pairs (BD PharMingen) for detecting IL-6, IL-1β, IL-12p40, and TNF-α.

Assessment of Mitochondrial DNA Content and Mass

For the quantification of mitochondrial content, we measured the mitochondrial (mt) to nuclear (n) DNA ratio. Pyruvate kinase was used as a marker for nDNA and NADH dehydrogenase subunit 1 (ND-1) for mtDNA. Real-time PCR reactions were performed according to the manufacturer's instructions (SYBR green PCR master mix; QIAGEN), and thermal cycling was performed in a Rotor gene 6000 instrument (QIAGEN). The mtDNA content was normalized to that of nuclear DNA. To evaluate mitochondrial mass, we stained the cells with MitoTracker Green FM (Life Technologies), which stains mitochondrial matrix protein, irrespective of the membrane potential, and thus provides an accurate assessment of mitochondrial mass. Fluorescence intensity was measured by flow cytometry and confocal laser-scanning microscopy, and the values were corrected for the total protein concentration.

Metabolic Assays

Lactate levels in the medium were determined using a Lactate Assay Kit (Biovision), according to the manufacturer's instructions. Briefly, the cells were stimulated with LPS for the indicated periods and the supernatants were then collected and stored at −80°C to inactivate lactate dehydrogenase. The reaction mix was added to the samples, which were then analyzed on a microplate reader (OD_{570nM}). The NAD^+/NADH ratio was measured from whole-cell lysates using a NAD^+/NADH Quantification Colorimetric Kit (BioVision), according to the manufacturer's instructions. For real-time analysis of the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR), BMDM were analyzed using an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). In brief, BMDM were plated in XF-24 cell culture microplates (2×10^5 cells/well in 200 μL) and then stimulated with LPS for the indicated time periods. At the indicated time points, the medium was removed, and the cells were washed and analyzed in XF Running Buffer (unbuffered RPMI, 10 mM glucose, 10% FCS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, and 20 ng/mL of GM-CSF) according to the manufacturer's instructions to analyze real-time values of OCR and ECAR. Where indicated, ECAR and/or OCR were analyzed in response to 2 μg/mL oligomycin, 5 μM CCCP, and 2 μM rotenone (all from Sigma-Aldrich).

Statistical Analyses

Differences between averages were analyzed using a two-tailed Student's t test. The significance of differences in survival was determined using a log-rank test.

ACCESSION NUMBERS

The raw DNA microarray data were submitted to the Gene Expression Omnibus under the accession number GSE58515.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.07.003.

AUTHOR CONTRIBUTIONS

J.-M.Y., T.S.K., H.-M.L., J.H., C.R.D., J.K.K., H.S.J., C.-S.Y., C.-H.L., G.R.K., H.-S.C., J.-M.V., D.D.M., V.G., and E.-K.J. designed and performed the experiments and analyzed and interpreted results; S.Y.K., J.K.K., and K.-S.P. performed all plasmid construction and contributed to the luciferase assay; T.S.K. assisted during bone marrow transplantation experiments; H.-S.C., and D.D.M. contributed the reagents; V.G. and E.-K.J. directed the study; J.-M.Y., V.G., and E.-K.J. wrote the manuscript, and all authors read and approved the manuscript.

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