# Nuclear receptor ERRα and coactivator PGC-1β are effectors of IFN-γ-induced host defense

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**Macrophage activation by the proinflammatory cytokine interferon-γ (IFN-γ) is a critical component of the host innate response to bacterial pathogenesis. However, the precise nature of the IFN-γ-induced activation pathway is not known. Here we show using genome-wide expression and chromatin-binding profiling that IFN-γ induces the expression of many nuclear genes encoding mitochondrial respiratory chain machinery via activation of the nuclear receptor ERRα (estrogen-related receptor α, NR3B1). Studies with macrophages lacking ERRα demonstrate that it is required for induction of mitochondrial reactive oxygen species (ROS) production and efficient clearance of *Listeria monocytogenes* (LM) in response to IFN-γ. As a result, mice lacking ERRα are susceptible to LM infection, a phenotype that is localized to bone marrow-derived cells. Furthermore, we found that IFN-γ-induced activation of ERRα depends on coactivator PGC-1β (peroxisome proliferator-activated receptor γ coactivator-1β), which appears to be a direct target for the IFN-γ/STAT-1 signaling cascade. Thus, ERRα and PGC-1β act together as a key effector of IFN-γ-induced mitochondrial ROS production and host defense.**

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Macrophages are the principal phagocytic cells in the immune system, and thus play a key defense role during bacterial infection. Upon infection, macrophages ingest bacterial pathogens through phagocytosis. Phagosomes capturing pathogens eventually mature into phago-lysosomes within which pathogens are destroyed. The antibacterial activities of macrophages are triggered by interferon-γ (IFN-γ) (Nathan et al. 1983), a proinflammatory cytokine that exerts its effects through activation of the JAK/STAT-1 pathway (Darnell et al. 1994) and transcriptional induction of antibacterial genes including nitric oxide synthase (iNOS) (MacMicking et al. 1995) and GTP-binding protein LRG-47 (MacMicking et al. 2003; Gutierrez et al. 2004). In addition, IFN-γ alters the expression of up to 10% of the genome in macrophages (Ehrt et al. 2001), although the contribution of most of the IFN-γ inducible genes to IFN-γ-induced host defense is largely unknown.

One of the genes whose expression in the macrophage was found to be inducible by IFN-γ was nuclear receptor ERRα (estrogen-related receptor α, NR3B1) (Barish et al. 2005), which belongs to a superfamily of ligand-activated transcription factors (Chawla et al. 2001). Historically, ERRα was the first orphan nuclear receptor to be identified and it shares a high degree of sequence homology with two estrogen receptors (ERα and ERβ), as well as two other ERR subfamily members (ERRβ and ERRγ)

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(Giguere et al. 1988; Giguere 2002). It can bind to consensus ER-binding sites in vitro, an inverted-repeat of the core element AGGTCA separated by three nucleotides, as a homodimer and is believed to have the potential to influence estrogen signaling pathways such as bone formation and breast cancer (Giguere 2002). It also binds to an extended consensus half site TCAAGGTCA or its variants found in the promoter of many genes involved in mitochondrial function (Sladek et al. 1997; Mootha et al. 2004; Schreiber et al. 2004; Dufour et al. 2007). Indeed, ERRα has been identified as a transcriptional regulator of MCAD, a key enzyme in mitochondrial fatty acid β-oxidation (FAO) (Sladek et al. 1997; Vega and Kelly 1997), and more recently has been shown to be a target for ligand-independent coactivators peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and PGC-1β and to control broad aspects of mitochondria biology including mitochondria biogenesis, FAO, and oxidative respiration (Huss et al. 2002; Kamei et al. 2003; Schreiber et al. 2003, 2004; Mootha et al. 2004; Wende et al. 2005, Dufour et al. 2007).

PGC-1α and a related protein, PGC-1β, have been identified as master regulators of mitochondrial oxidative metabolism (Lin et al. 2005; Finck and Kelly 2006). They both activate all of the ERR subtypes as well as other transcription factors implicated in mitochondrial gene regulation such as PPARα, PPARδ, nuclear respiratory factor-1, and GABPa/b. In addition, a variety of other transcriptional factors have been proposed to mediate biological activity elicited by PGC-1 proteins. Consistent with their role as a regulator of oxidative metabolism, they are expressed most highly in oxidative tissues, such as brown adipose tissue, cardiac and skeletal muscles, kidney, and brain. Thus, knockout (KO) mice for PGC-1α or PGC-1β exhibit a variety of metabolic defects (Lin et al. 2004; Leone et al. 2005; Lelliott et al. 2006; Vianna et al. 2006; Sonoda et al. 2007). In addition, expression of PGC-1β in macrophages was recently reported, although its physiological importance in this cell type is not well understood (Vats et al. 2006).

A few studies have linked mitochondrial reactive oxygen species (ROS) production in modulation of macrophage-mediated innate immune function. UCP-2 is a ROS-activated mitochondrial uncoupling protein believed to function to reduce mitochondrial ROS production by releasing membrane potential (Echtay et al. 2002). UCP-2 overexpression in macrophages suppresses lipo-polysaccharide-induced intracellular ROS accumulation (Kizaki et al. 2002). UCP-2-deficient mice produce an increased level of ROS in macrophages and are resistant to infection of intracellular pathogens *Toxoplasma gondii* (Arsenijevic et al. 2000) and *Listeria monocytogenes* (LM) (Rousset et al. 2006). However, whether mitochondrial ROS production is a regulated process during infection is not clear.

In this report, we show that ERRα and PGC-1β act as downstream effectors for IFN-γ, regulating mitochondrial output in macrophages. The loss of ERRα or PGC-1β results in decreased mitochondrial gene expression, intracellular ROS level, and bacterial clearance in IFN-γ-activated macrophages. These results not only uncover a previously unappreciated downstream effect elicited by IFN-γ, but also suggest a link between mitochondrial oxidative metabolism and macrophage-driven antibacterial immunity.

## Results

### *ERRα mediates cytokine-induced mitochondrial gene expression in macrophages*

We have previously generated mice deficient for ERRα (or Esrra; ERRα KO mice) (Luo et al. 2003). When macrophages were differentiated from bone marrow cells of wild-type and ERRα KO mice and activated by prototypical cytokines, IFN-γ or interleukin-4 (IL-4), no defects were observed in the expression of surface marker or activation marker genes in ERRα KO cells (Supplementary Figs. S1, S2). To identify the role of ERRα in macrophage function, the global mRNA expression profiles were determined using oligo DNA microarray analyses. Gene Ontology (GO) analysis showed that expression of "mitochondrion" and related pathways was significantly altered in ERRα KO cells in both IFN-γ- and IL-4-treated but not in mock-treated groups, suggesting that ERRα specifically controls inducible mitochondrial function associated with activated macrophages (Fig. 1A). Furthermore, of the genes whose expression is significantly higher in wild-type cells than ERRα KO cells in both IFN-γ- and IL-4-treated groups, the majority (74 of 86) were found to encode mitochondrial proteins (Fig. 1B; Supplementary Fig. S3; Supplementary Tables S1–S3). In particular, 47 genes are directly involved either in the mitochondrial tricarboxylic acid (TCA) cycle or oxidative phosphorylation (OXPHOS). Hierarchical clustering analysis and quantitative PCR (Q-PCR) analysis indicated that the expression of these genes do not differ between wild-type and ERRα KO cells in the resting state, but are induced by IFN-γ and IL-4 in an ERRα-dependent manner (Fig. 1C,D; Supplementary Figs. S4, S5).

To determine whether ERRα directly regulates its downstream target genes, chromatin immunoprecipitation (ChIP) assays were employed. We found that ERRα binds the target promoters even in the absence of the activating cytokines, but the binding was typically augmented in the presence of IFN-γ (Supplementary Fig. S6). Consistently, ERRα protein was constitutively found in the nucleus, and was increased by IFN-γ or IL-4 treatment (Supplementary Fig. S7). To further determine whether ERRα directly regulates its target genes, we carried out an unbiased genome-wide mapping of ERRα-binding sites in IFN-γ treated macrophages using ChIP followed by hybridization to a genomic DNA microarray containing the −800 to +200 regions of ~19,000 promoters. We identified a total of 215 promoters ($P < 0.01$) bound by ERRα in the presence of IFN-γ, spanning various functional groups (Supplementary Tables S4, S5; Supplementary Fig. S8). A comparison of data from ChIP and gene expression analyses on IFN-γ treated macro-

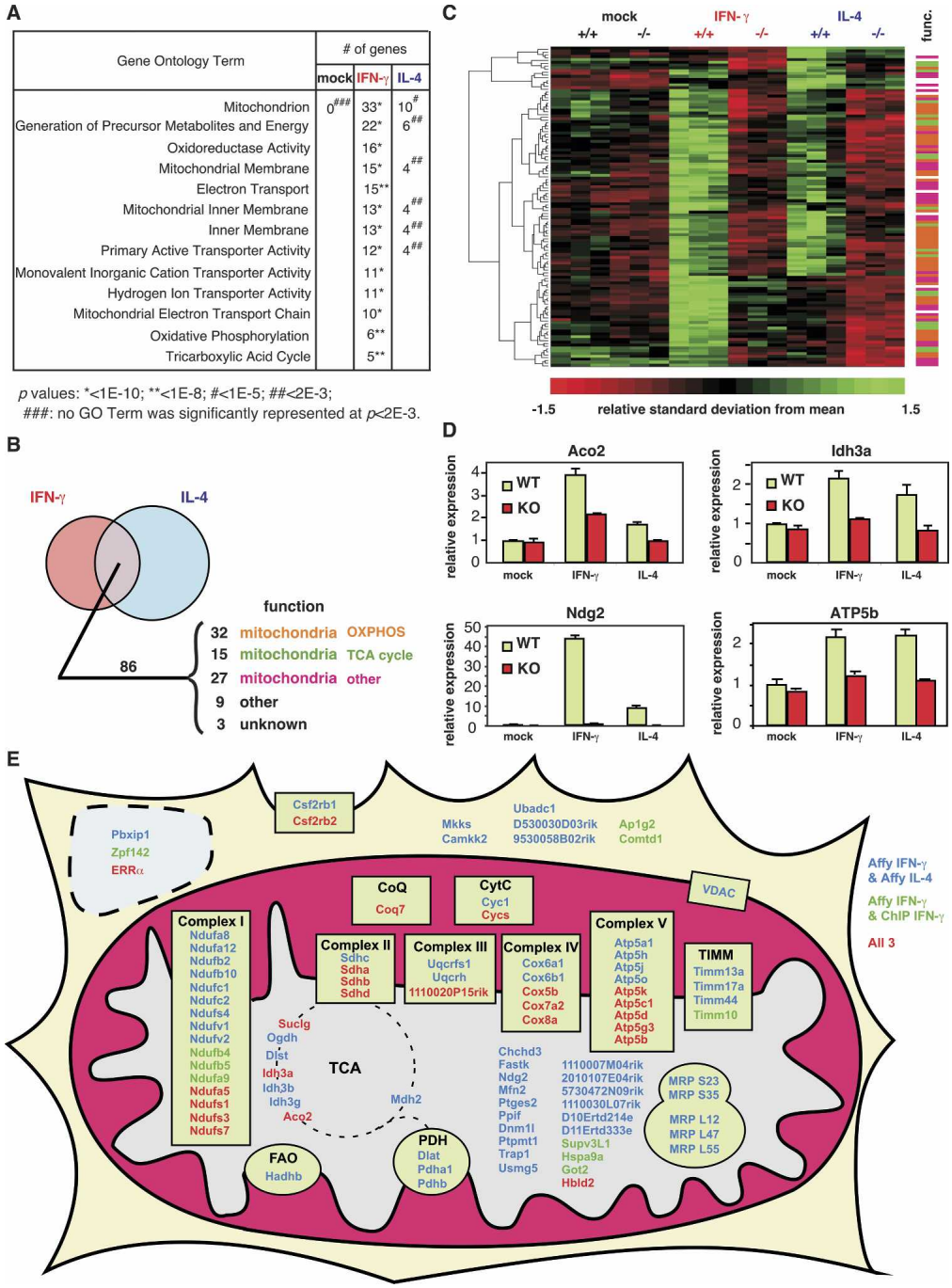


**Figure 1.** ERRα-dependent induction of nuclear-encoded mitochondrial genes by IFN-γ or IL-4. (*A*) GO pathways whose expression were significantly different between wild type and ERRα KO and the number of genes identified for each pathway. (*B*) Functional grouping of 86 unique genes whose expression are suppressed both in IFN-γ- or IL-4-treated ERRα KO macrophages compared with wild-type macrophages. (*C*) The relative expression levels of 86 genes identified in *B* are indicated by representative colors as shown at the *bottom*. The assigned function for each feature is indicated at *right* by color as shown in *B*. (*D*) Q-PCR mRNA expression analysis in macrophages ($n = 4$) treated with the indicated cytokines for 12 h. *Aco2*, *Idh3a*, *Atp5b*, and *Ndg2* encode mitochondrial proteins. (*E*) ERRα targets identified by genome-wide expression and chromatin-binding profiling. Functional locations (nucleus, plasma membrane, cytoplasm, or mitochondria) of the identified gene products are schematically shown. (Blue) Common between the expression profiling of IFN-γ- and IL-4-treated cells (*B*); (green) common between expression profiling and chromatin-binding profiling of ERRα in IFN-γ-treated cells (Supplementary Fig. S8); (red) common for both of these two criteria. (MRP) Mitochondrial ribosomal proteins; (PDH) pyruvate dehydrogenase complex; (TIMM) translocase of inner mitochondria membrane.

phages indicated that many of the genes that are positive for both analyses are involved in mitochondrial respiration; the majority of which were independently identified by IFN-γ/IL-4 comparison of the transcriptome (Fig. 1E; Supplementary Fig. S8). Together, these results identify ERRα as a direct effector of IFN-γ- and IL-4-induced mitochondrial gene expression in macrophages.

### *ERRα controls mitochondrial function and bacteria clearance in activated macrophages*

Functional studies further indicated that the loss of ERRα mimics a mild mitochondrial dysfunction in the activated macrophages. Cellular oxygen consumption was increased by 52% in wild-type macrophages 36 h after addition of IFN-γ, compared with mock-treated cells; this response was lost in ERRα KO macrophages (Fig. 2A). Although mitochondrial biogenesis did not accompany the increase in respiration (Supplementary Fig. S9), inhibition by a specific complex IV inhibitor, potassium cyanide (KCN), indicated that the observed cellular oxygen consumption is dependent on mitochondria, and not by the so-called "respiratory burst" (Fig. 2A, right panel). In agreement, mitochondrial membrane potentials were 15% higher in activated wild-type cells compared with activated ERRα KO cells as assessed using Rhodamine-123 staining (Fig. 2B). As mitochondrial respiration is a source of ROS, the intracellular ROS levels were 45% higher in activated wild-type cells compared with activated ERRα KO cells as assessed using a ROS-activated fluorescent dye, dichlorofluorescin (DCF) (Fig. 2C,D). Conversely, expression of ERRα protein fused to the constitutive transcriptional activator VP16 is sufficient to increase ROS by 66% in the macrophage cell line, RAW264 (Supplementary Fig. S10). In contrast, IgG-induced ROS production through NADPH oxidase (respiratory burst) appeared normal in IFN-γ-pretreated ERRα KO cells (Fig. 2E). Rotenone (complex I inhibitor) or KCN had a similar effect on ROS levels even at the

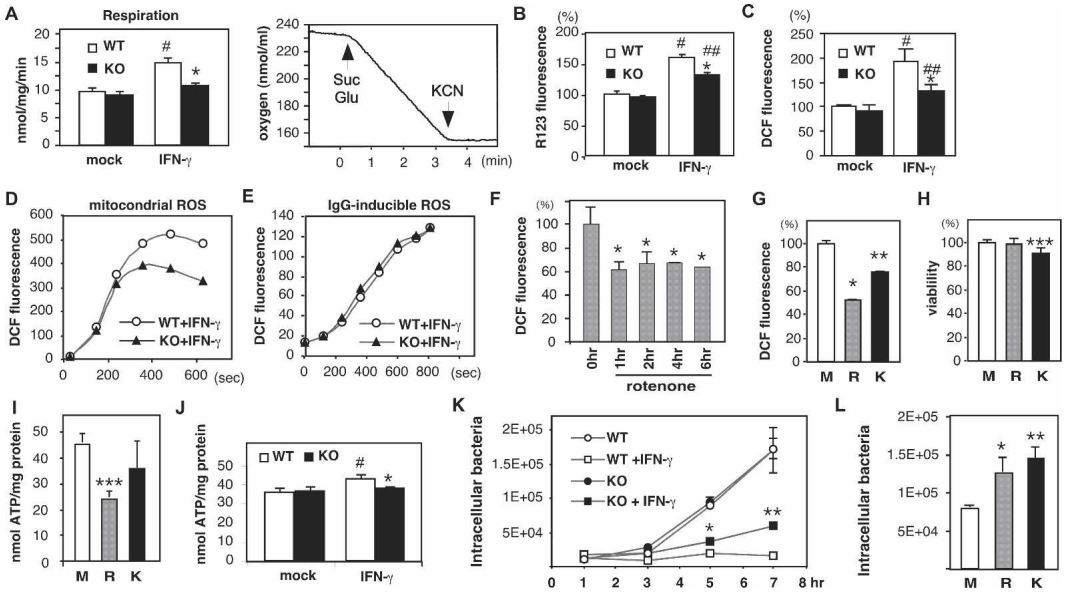


**Figure 2.** IFN-γ induces mitochondrial ROS production and LM clearance in macrophages through ERRα. (*A*, *left*) Cellular $O_2$ consumption in digitonin-permeabilized macrophages ($n = 6$) cultured with or without IFN-γ for 36 h. (*) $P < 0.002$ (wild type vs. ERRα KO); (#) $P < 0.004$ (IFN-γ vs. mock-treated group). (*Right*) A representative oxygraph pattern. (Arrows) Addition of substrates (succinate and glutamate) or the inhibitor (KCN). (*B*) Macrophages were cultured with or without IFN-γ for 36 h, then cultured in medium containing Rhodamine-123 (R123) for 30 min, harvested, and subjected to FACS analysis, to determine the mitochondrial electron potential. The results represent relative median values of the fluorescence ($n = 6$). (*) $P < 0.005$ (wild type vs. ERRα KO); (#) $P < 5E\text{-}6$; (##) $P < 1E\text{-}8$ (IFN-γ vs. mock-treated group). (*C*) Macrophages were cultured with or without IFN-γ for 36 h, then cultured in medium containing DCF for 30 min, harvested, and subjected to FACS analysis to determine the intracellular ROS level. The results represent relative median values of the fluorescence ($n = 3$. (*) $P < 0.01$ (wild type vs. ERRα KO); (#) $P < 0.001$; (##) $P < 0.05$ (IFN-γ vs, mock-treated group). (*D*) The rate of DCF oxidation was determined by adding DCF ($t = 0$) to suspensions of IFN-γ-activated macrophages followed by FACS analysis at the indicated time points. Median fluorescence was plotted for each measurement. (*E*) A similar experiment as *C*, except IgG-conjugated DCF was used to measure the intracellular ROS level generated by NADPH oxidase. (*F*) The effects of rotenone (50 nM) on the intracellular ROS level in IFN-γ-activated macrophages. (*) $P < 0.01$. (*G–I*) The ROS level (*F*), viability (*G*), and total cellular ATP content (*H*), of IFN-γ-activated macrophages after 6 h of treatment with no drug (M), with 50 nM rotenone (R), or with 1 mM KCN (K). (*) $P < 3E\text{-}05$; (**) $P < 3E\text{-}04$; (***) $P < 0.03$. (*J*) Total cellular ATP content in IFN-γ-activated macrophages. (*) $P < 0.03$ (wild type vs. KO); (#) $P < 0.03$ (mock vs. IFN-γ-treated). (*K*) In vitro LM infection of resting and activated macrophages. (*) $P < 0.02$; (**) $P < 0.0003$ (wild type vs. KO). (*L*) A similar in vitro LM infection of activated macrophages. After infection, IFN-γ-pretreated cells were incubated for 6 h without mitochondrial inhibitor (M), with 50 nM rotenone (R), or with 1 mM KCN (K). (*) $P < 0.05$; (**) $P < 0.01$.

subtoxic concentrations used for the study (Fig. 2F–H). The intracellular ATP level was also dramatically reduced by rotenone in the activated macrophages (Fig. 2I). However, the loss of ERRα had little effect on the intracellular ATP level (Fig. 2J). This difference is presumably because the drugs inhibit both the basal and IFN-γ-induced respiration, whereas the loss of ERRα compromises only the latter.

An increase in mitochondrial ROS production has been suggested to enhance resistance against intracellular pathogens (Arsenijevic et al. 2000; Rousset et al. 2006). To determine whether the observed ROS induction by IFN-γ contributes to pathogen resistance, we infected wild-type and ERRα KO macrophages with the Gram-positive bacterial pathogen LM and quantified the viable intracellular bacteria. In the absence of IFN-γ treatment, we did not detect a significant difference between wild-type and ERRα KO macrophages (Fig. 2K). However, IFN-γ increased LM clearance by wild-type macrophages as previously described (Portnoy et al. 1989), and this response was compromised in ERRα KO macrophages (Fig. 2K). This ERRα-dependent bactericidal activity was also observed in the presence of specific inhibitors for iNOS or NADPH oxidase (Supplementary Fig. S11), suggesting that ERRα may function through an iNOS and NADPH oxidase-independent pathway. Furthermore, rotenone and KCN increased intracellular bacteria counts, supporting the idea that mitochondrial function is critical for IFN-γ-induced bactericidal activity (Fig. 2L).

### *ERRα KO mice are susceptible to* Listeria *infection*

The in vivo role of the ERRα-mediated pathway was further tested by intravenous (i.v.) LM infection. When exposed to bacteria ($2 \times 10^4$ colony forming units [cfu]), the number of LM in the liver and the spleen was increased >10-fold in ERRα KO mice compared with wild-type mice at day 2 post-infection (Fig. 3A,B). When a sublethal dose of LM ($1 \times 10^4$ cfu) was injected, ERRα KO mice showed increased splenomegaly at day 10 post-infection (Fig. 3C). Hematoxylin and eosin (H&E) staining indicated that the structure of the periarteriolar lymphocyte sheath (PALS) is overtly destroyed in the spleens of infected ERRα KO mice, whereas in the wild type, the PALS structures were largely intact (Fig. 3D). Several lines of evidence suggest that the defect in ERRα KO mice is not due to a failure to mount an activated macrophage response. First, serum cytokine levels (Fig. 3E; Supplementary Fig. S12) and gene expression in the spleen (Supplementary Fig. S13) and peritoneal macrophages (Fig. 3F) indicated normal cytokine production in the infected ERRα KO mice. Second, the relative numbers of macrophages, NK cells, and lymphocytes in the spleen of the infected KO mice were normal, except an increased lymphocyte population positive for an early activation marker, CD69, suggesting an intact immune system (Supplementary Table S6). Thus, the defect is not due to alterations in regulatory cytokines or compositional defects in the immune system, but rather due to a downstream pathway regulated by ERRα. Remarkably, this defect in the ERRα KO resulted in impaired survival upon exposure to LM when compared with wild-type mice (Fig. 3G). Using bone marrow chimeras, we localized the susceptibility of ERRα KO mice to LM infection to bone marrow-derived cells (Fig. 3H,I).

### *ERR coactivator PGC-1β is an immediate downstream target of IFN-γ*

Establishing the role for ERRα in mediating IFN-γ-induced mitochondrial gene network, we then sought to determine the mechanism whereby IFN-γ activates ERRα. Two related coactivator proteins, PGC-1α and PGC-1β, have been previously identified as ligand-independent coactivators for ERRα (Lin et al. 2005; Finck and Kelly 2006). We failed to detect PGC-1α expression in murine macrophages with or without cytokine treatment (data not shown). However, PGC-1β expression was detectable in macrophages and was further induced by IFN-γ and IL-4, but not by six other cytokines, which correlated with induction of ERRα target genes (Fig. 4A; Suplementary Fig. S4). IFN-γ and IL-4 transduce their activities by activating transcription factors STAT-1 and STAT-6, respectively (Darnell et al. 1994). The PGC-1β induction by IFN-γ was lost in STAT-1-deficient macrophages and so were ERRα target genes (Supplementary Fig. S14). Furthermore, PGC-1β expression was sufficient to activate ERRα in RAW264 cells (Fig. 4B), suggesting the likely involvement of PGC-1β in cytokine-induced ERRα activation.

Inspection of the murine PGC-1β gene promoter sequence identified a perfect consensus sequence for STAT-1 binding (GAS for IFN-γ activation sequence), which is conserved in rat, dog, and human genomes (Fig. 4C; Seidel et al. 1995). We also identified a potential STAT-6-binding site (4AS for IL-4 activation sequence), although it was only found in the mouse promoter, but not in the three other species. Several lines of evidence suggested that these sites likely act as functional STAT-binding sites. First, ChIP assay indicated that STAT-1 binds to this region upon IFN-γ treatment (Fig. 4D). Second, electrophoretic mobility shift assay (EMSA) using macrophage nuclear extracts indicated that IFN-γ and IL-4 induce binding activities to the PGC-1β GAS and 4AS sites, respectively (Fig. 4E). A specific antibody was used to demonstrate that the IFN-γ-induced GAS-binding activity contains STAT-1 protein (Fig. 4E). Finally, PGC-1β GAS or 4AS elements can mediate cytokine-activated gene expression in transfected RAW264 cells (Fig. 4F).

### *PGC-1β mediates cytokine-induced activation of ERRα and mitochondrial function*

To determine whether PGC-1β mediates the IFN-γ-induced ERRα activation, macrophages were derived from bone marrow of PGC-1β-deficient or control wild-type mice (Sonoda et al. 2007), activated by IFN-γ or IL-4, and

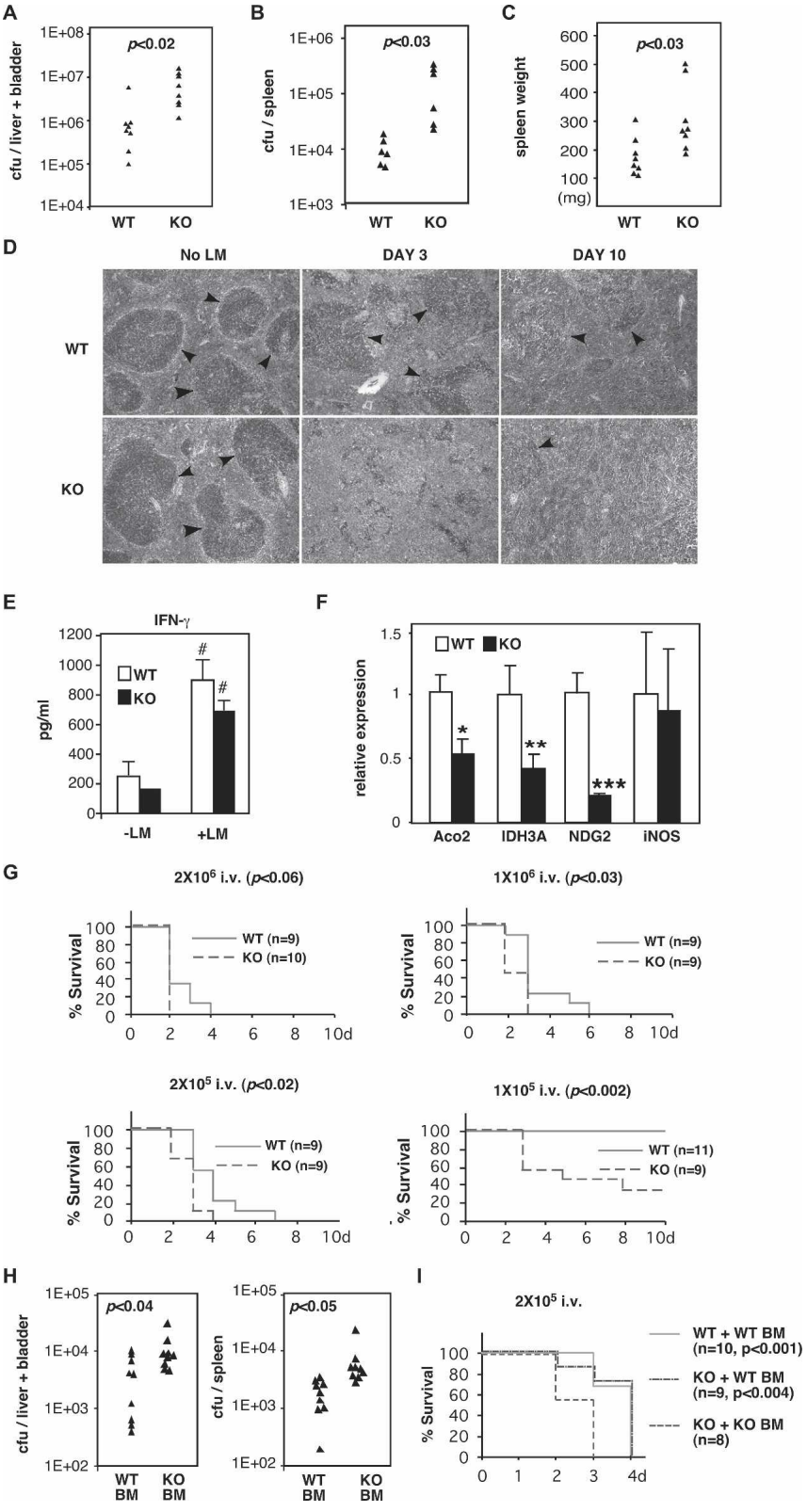


**Figure 3.** ERRα KO mice are defective for LM clearance. (*A*,*B*) Bacteria counts of the whole liver and gallbladder ($n = 8$) (*A*) and the spleen ($n = 6$) (*B*) at day 2 post-$2 \times 10^4$ LM i.v. injection. (*C*) Spleen weights at day 10 post-$1 \times 10^4$ LM i.v. injection ($n = 8$). (*D*) H&E straining of spleens after $1 \times 10^4$ LM infection. (Arrowheads) The PALS structures. (*E*) Serum IFN-γ concentrations at day 2 post-infection ($2 \times 10^4$ cfu). No significant difference between wild type and KO. (#) $P < 0.05$ (–LM vs. +LM). (*F*) Q-PCR analysis of peritoneal exudates cells ($n = 6$) at day 4 post-LM infection ($1 \times 10^6$ cfu). (*) $P < 0.02$; (**) $P < 0.04$; (***) $P < 0.0004$. Note that expression of ERRα target genes, but not of iNOS, is significantly reduced in the KO cells. (*G*) Kaplan-Meier survival plot after i.v. injection of the indicated dose of LM. (*H*) Bacteria counts of the whole liver and gallbladder (*left*) and the spleen (*right*) ($n = 10$) at day 2 post-$2.4 \times 10^4$ LM i.v. injection. (*I*) Kaplan-Meier survival plot for bone marrow-transplanted mice after $2 \times 10^5$ LM i.v. injection. *P* values shown are of KO + KO BM to the indicated group.

gene expression were determined by Q-PCR. Like ERRα KO macrophages, no defects for differentiation or activation were observed in PGC-1β KO cells (Supplementary Figs. S15– S17). However, induction of ERRα target genes by IFN-γ or IL-4 was blunted in PGC-1β KO cells (Fig. 5A). Oligo DNA microarray analyses further

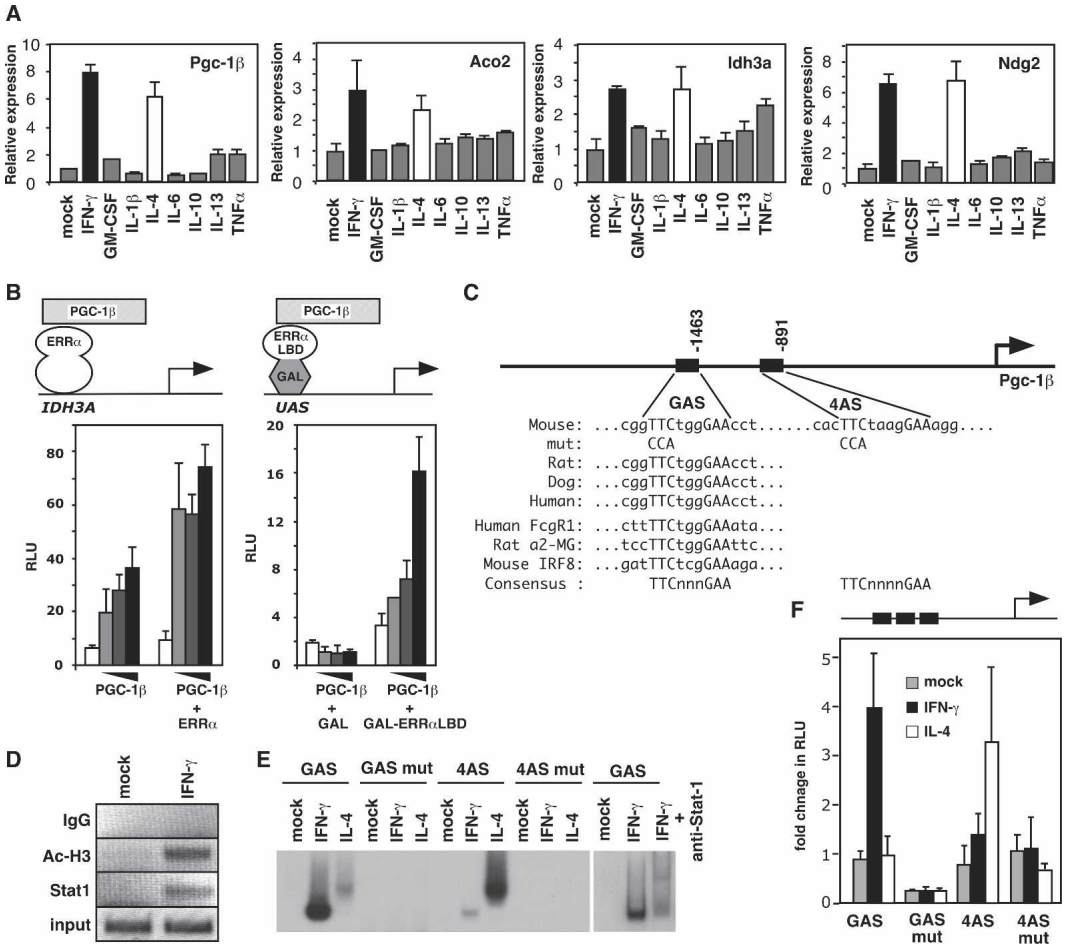


**Figure 4.** PGC-1β expression is regulated by IFN-γ and correlates with ERRα activation. (*A*) Q-PCR mRNA expression analysis in macrophages ($n = 4$) treated with the indicated cytokines for 12 h. (*B*) Transient transfection reporter assay of murine macrophage cell line RAW264. Schematic diagrams for the experiments are shown at the *top*. (*Left*) Cells were cotransfected with an expression vector for mouse PGC-1β and/or mouse ERRα, together with a luciferase reporter harboring three copies of ERRα-binding site from the murine *Idh3a* gene. (*Right*) Cells were cotransfected with an expression vector for mouse PGC-1β and/or human ERRα ligand-binding domain fused to GAL4 DNA-binding domain (GAL-ERRαLBD), together with a luciferase reporter harboring three copies of GAL4-binding site (UAS). The results represent the average of triplicate experiments for luciferase activity normalized by β-gal activity. (*C*) Schematic diagram of murine PGC-1β promoter. (GAS) IFN-γ activation sequence; (4AS) IL-4 activation sequence. (*D*) ChIP assay using anti-STAT-1 or anti-acetylated histone H3 antibody in IFN-γ-activated macrophages. (*E*) EMSA was used to test specific binding of IFN-γ- or IL-4-induced nuclear activities to the potential GAS and 4AS from the PGC-1β promoter. Supershift with anti-STAT-1 antibody shows that the IFN-γ-induced binding complex contains STAT-1. (*F*) Transient transfection reporter assay of RAW264 cells with luciferase reporter driven by three copies of GAS and 4AS sites. After the transfection, cells were treated with the indicated cytokines for 12 h. The results represent the average of triplicate experiments for normalized luciferase activity.

showed that expression of almost all the ERRα target genes identified by the global mRNA expression profiling (Fig. 1) was reduced in activated PGC-1β KO cells compared with wild-type cells (Fig. 5B). Indeed, gene expression profile of PGC-1β KO cells was almost identical to that of ERRα KO cells, and we found no genes that were regulated only by PGC-1β or by ERRα. Consistent with the alteration in gene expression, the loss of PGC-1β phenocopies the loss of ERRα; IFN-γ-activated PGC-1β-deficient macrophages show a reduced membrane potential (Fig. 5C), a reduced ROS level (Fig. 5D), and an increased number of viable LM when infected in vitro (Fig. 5E) compared with wild-type cells. When PGC-1β-deficient mice were exposed to LM, they show increased numbers of viable LM in the liver and the spleen (Fig. 5F), and an increased mortality compared with wild-type controls (Fig. 5G). In aggregate, these results indicated that PGC-1β is a direct target of the IFN-γ/STAT-1 pathway that mediates IFN-γ-induced ERRα activation, mitochondrial ROS production, and pathogen clearance.

## Discussion

PGC-1β and ERRα previously have been shown to regulate broad aspects of mitochondrial biology, including FAO, mitochondrial biogenesis, oxidative phosphoryla-

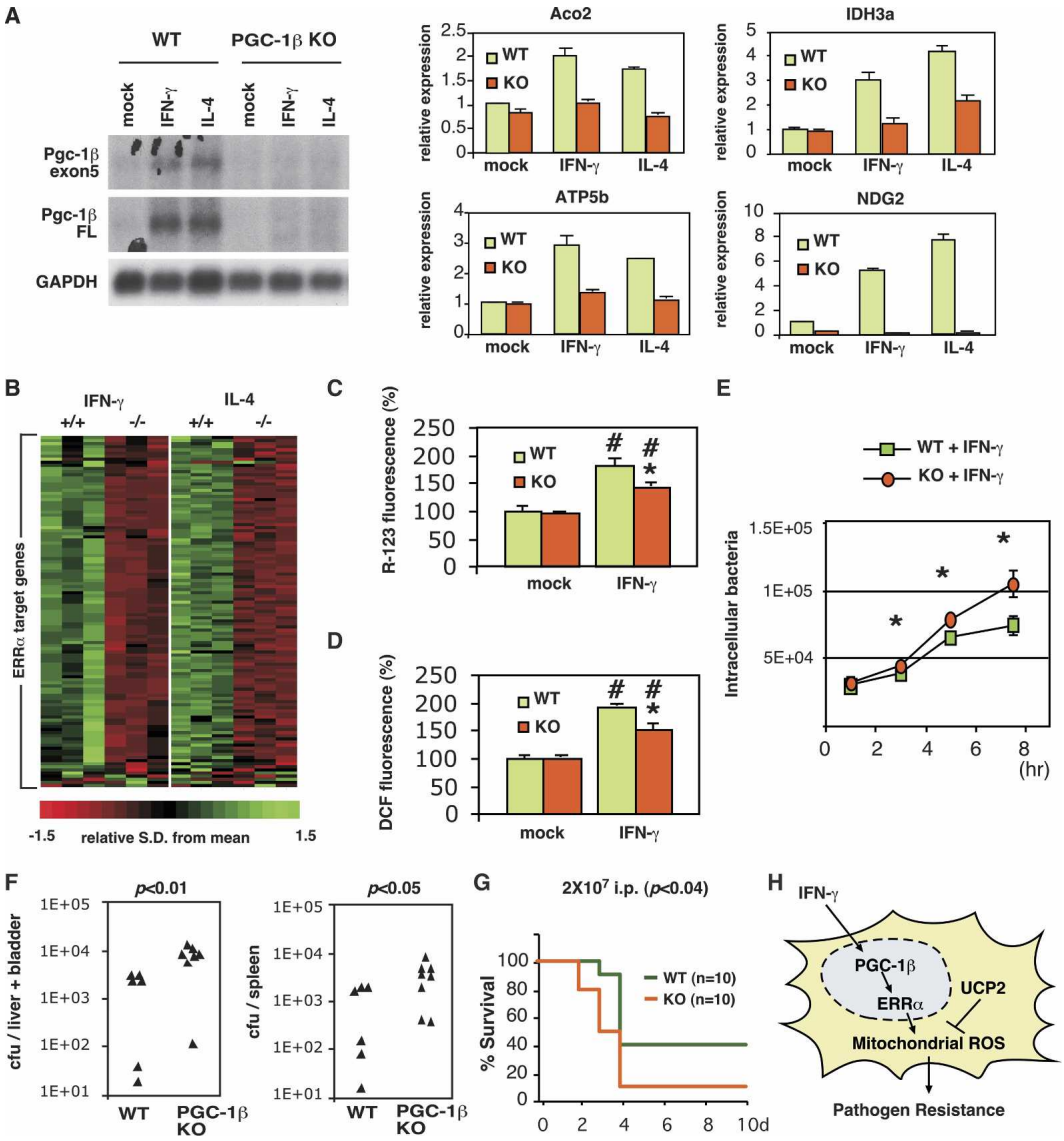


**Figure 5.** PGC-1β KO mice are defective for LM clearance. (*A*) Northern blot (*left*) and Q-PCR (*right*) mRNA expression analyses in PGC-1β-deficient macrophages activated with the indicated cytokines. (*Left*) cDNA encoding a part of exon 5 (*top*) or entire PGC-1β (*middle*) was used as probe. (GAPDH) Loading control. (*B*) The relative expression levels of 86 genes identified in Figure 1B in wild-type and PGC-1β KO cells are indicated by colors as shown at the *bottom*. (*C*,*D*) IFN-γ-activated macrophages were cultured in medium containing Rhodamine-123 (*C*) or DCF (*D*) for 30 min, harvested, and subjected to FACS analysis to determine the mitochondrial membrane potential (*C*) and the intracellular ROS level (*D*), respectively. The results represent relative median values of the fluorescence ($n = 4$). (*) $P < 0.05$ (wild type vs. ERRα KO); (#) $P < 0.05$ (IFN-γ vs. mock-treated group). (*E*) In vitro LM infection of activated macrophages. (*) $P < 0.05$ (wild type vs. KO). (*F*) Bacteria counts of the whole liver and gallbladder (*left*) and the spleen (*right*) ($n = 6$–$8$) at day 3 post-$2 \times 10^6$ LM i.p. injection. (*G*) Kaplan-Meier survival plot after injection of the indicated dose of LM. (*H*) A model for the role of mitochondria ROS in pathogen resistance. Disruption of ERRα impairs inducible mitochondrial ROS production and pathogen resistance (this study), whereas disruption of mitochondrial uncoupling protein (UCP2), whose function is to reduce mitochondrial ROS (Echtay et al. 2002), results in constitutive pathogen resistance (Arsenijevic et al. 2000; Rousset et al. 2006).

tion in fat, liver, heart, and skeletal muscle as well as brown fat-dependent thermogenesis (Sladek et al. 1997; Vega and Kelly 1997; Huss et al. 2002; Kamei et al. 2003; Luo et al. 2003; Schreiber et al. 2003, 2004; Mootha et al. 2004; Wende et al. 2005; Dufour et al. 2007; Sonoda et al. 2007; Villena et al. 2007). Our study using macrophages lacking ERRα or PGC-1β provide further genetic evidence for the role for these factors as central regulators of oxidative energy metabolism. Of all the mitochondrial genes identified by our combination of mRNA expression and chromatin-binding profiling, the majority encodes components of the TCA cycle, the electron transport chain or other basic mitochondrial functions. Indeed, ERRα- or PGC-1β-deficient macrophages showed lower mitochondrial respiratory activity and intracellular ROS levels compared with wild-type controls.

In addition to characterized mitochondrial components, our experiments also identified a number of potential ERRα target genes whose protein products though localized to mitochondria have not been linked to a particular function, leaving the question as to whether or not these genes are also involved in mitochondrial energy metabolism. One gene of particular interest is *Ndg2*, which is the most robustly regulated gene by ERRα and PGC-1β in macrophages. *Ndg2* was originally identified as a transcriptional target of nuclear receptor Nur77 in thymocytes (Rajpal et al. 2003) and encodes a protein similar to a bacterial protein required for sodium-coupled ATP synthesis (Buckel 2001). Somewhat surprisingly, the FAO pathway, which is thought to be a major pathway regulated by PGC-1 and ERRα in muscle or liver, does not appear to be significantly regulated through PGC-1β or ERRα in our macrophage system. This may be due to the scarcity of PPARα expression in murine macrophages (Li et al. 2004; Barish et al. 2005), which was recently shown to mediate FAO induction by ERRα overepression in primary fibroblasts (Huss et al. 2004). Whether IFN-γ, PGC-1β, and ERRα affect FAO in human macrophages that express PPARα at a higher level is an open question that needs to be addressed in the future.

Our experiments with LM suggest that PGC-1β and ERRα constitute a key component of the innate immune response against intracellular bacterial pathogens. In our current model, they act together as a molecular switch to promote mitochondrial function during macrophage activation while limiting unwanted ROS production in the resting state (Fig. 5H). This "transcriptional switch" is activated by IFN-γ through direct control of PGC-1β expression via the conserved GAS element (STAT-1-binding site) in the PGC-1β promoter. This model links the IFN-γ signaling pathway to mitochondrial ROS production, which has previously been proposed to increase resistance to intracellular pathogens (Fig. 5H; Arsenijevic et al. 2000; Rousset et al. 2006). Consistent with our model, the level of intracellular ROS, but not ATP, correlates with the bacterial clearance, suggesting that ROS production is likely to be the causative compromise to INF-γ protection in ERRα- or PGC-1β-deficient macrophages, although further study is required to elucidate the mechanism by which mitochondrial ROS promotes bacteria clearance.

In addition to IFN-γ, an anti-inflammatory Th2 cytokine, IL-4, that induces alternative macrophage activation in response to parasitic and extracellular pathogens (Herbert et al. 2004) induces PGC-1β expression (Vats et al. 2006) and activates ERRα (Fig. 1). The alternative Th2 activation involves antigen presentation, which can also be promoted by ROS at least in resident hepatic macrophages (Kupffer cells) (Maemura et al. 2005). Therefore, ERRα-mediated induction of the mitochondrial pathway may also play a role during parasite infection. The alternative Th2 activation pathway has recently been shown to depend on a PGC-1β-dependent induction of FAO and mitochondrial biogenesis (Vats et al. 2006). This seems to differ from our observations as loss of PGC-1β did not result in detectable defects in IL-4-induced activation in our in vitro primary culture system (Supplementary Figs. S16, S17; data not shown). Furthermore, the loss of ERRα (or PGC-1β) in macrophages does not alter the expression of FAO genes or change mitochondrial DNA levels (Supplementary Figs. S4, S5, S9, S16). Perhaps, dependent on the conditions, PGC1-β activates other transcription factors, such as PPARδ, to mediate increased FAO and mitochondrial biogenesis during the alternative activation (Lee et al. 2006).

In summary, our results demonstrate a role for PGC-1β and ERRα in macrophages as downstream effectors of IFN-γ. While IFN-γ is approved for use in reducing the severity of infections as well as for the treatment of severe malignant osteopetrosis, its widespread actions limit its use due to severe side effects. Synthetic ERRα agonists that are currently under exploratory development for amelioration of type 2 diabetes and other metabolic disorders may thus serve as a potentially more beneficial approach in the amelioration of a variety of bacterial and parasitic infections as well.

## Materials and methods

### *Animals*

ERRα KO mice and PGC-1β in a C57B/6J background were described previously (Luo et al. 2003; Sonoda et al. 2007). In each case, male KO mice and age-matched wild-type controls from heterozygote crosses were used. Mice were maintained in a pathogen-free animal facility under standard 12 h light/12 h dark cycle, with access to chow and water ad libitum. LM infection was carried out using the 10403S strain as described (Portnoy et al. 1989; Joseph et al. 2004).

### *Cell culture and in vitro assays*

Bone marrow-derived macrophages were isolated and differentiated in the presence of M-CSF as described previously (Lee et al. 2003), and were subsequently cultured in RPMI medium containing 10% fetal bovine serum (FBS) and 0.2% glucose or galactose. For in vitro functional analysis, macrophages were precultured in the presence of 10 ng/mL IFN-γ for 36–42 h. In some cases, M-CSF was also included in medium. Cos7 cells and murine macrophage cell line RAW264 were cultured in DMEM medium containing 10% FBS. Recombinant mouse cytokines were from BD Pharmingen, except for M-CSF (R&D Systems).

Mitochondrial membrane potential was determined by incubating macrophages with 0.1 pg/mL Rhodamine-123 (Invitrogen) for 30 min followed by FACS analysis. ROS production was determined by incubating macrophages with 25 μM dichlorofluorescein-diacetate (Sigma-Aldrich) or oxyburst reagent (Molecular Probes) for the specified times followed by FACS analysis. Propidium iodide was used to distinguish dead cells. All the FACS analyses were conducted using FACS Scan (Becton Dickinson) and CellQuest software (Becton Dickinson). Cellular oxygen consumption rates were measured using a polarographic $O_2$ electrode (Oxygraph system, Hansatech Instruments) as described previously (Potluri et al. 2004). In vitro LM infection was performed as described (Portnoy et al. 1989; Joseph et al. 2004). Cellular ATP concentrations in total macrophage cellular extracts were determined using ATP Bioluminescence Assay

Kit CLS II (Roche). Transient transfections were carried out using Fugene HD (Roche). Serum cytokine levels were determined using a Bio-Plex cytokine assay kit on a Bio-Plex suspension array system (Bio-Rad). Recombinant cytokines were used as standards.

*Gene expression analysis and Q-PCR*

Total RNA was prepared with Trizol (Invitrogen) according to the manufacturer's instructions. For DNA microarray analysis, labeled Poly(A) RNA ($n = 3$) prepared from bone marrow-derived macrophages treated with 10 ng/mL IFN-γ or 10 ng/mL IL-4 for 12 h was hybridized to Affymetrix mouse genome 430A 2.0 arrays. Background correction, normalization, and expression values were obtained for 22,690 probe sets using RMA. Subsequently, the VAMPIRE microarray analysis suite (http://biome.sdsc.edu:8090/vampire) was used for GO (Hsiao et al. 2005). Independently, probe sets with an average log base 2 expression >6.1 were used to find significant differences by Student *t*-test ($P < 0.05$). Genes encoding mitochondria protein were found to be enriched in a group that was significantly down-regulated by at least 1.1-fold in both IL-4- and IFN-γ–treated ERRα KO samples. Typically, higher fold induction was observed by Q-PCR than by our Affymetrix analysis. All calculations were done using the R statistical environment (http://www.r-project.org) and packages from Bioconductor (http://www.bioconductor.org). Standard protocols were used for Q-PCR using SYBR Green dye (Invitrogen) with 36B4 as a standard. The entire microarray data is available at http://www.nursa.org/10.1621/datasets.04004. The sequence of primers used for mRNA expression will be provided on request.

*Genome-wide ChIP and location analysis*

After IFN-γ treatment (10 ng/mL for 12 h), macrophages were fixed with 1% final concentration formaldehyde for 10 min at room temperature, rinsed, and harvested with 1× PBS. The resultant cell pellet was sonicated, and protein–DNA complexes were enriched by immunoprecipitation with the ERRα-specific antibody (Laganiere et al. 2003); beads were added and washed as described previously (Laganiere et al. 2005). After de-cross-linking, the enriched DNA was repaired with T4 DNA polymerase (New England Biolabs) and ligated with linkers, as described (Odom et al. 2004). DNA was amplified using ligation-mediated PCR (LM-PCR), and then fluorescently labeled using BioPrime Array CGH genomic labeling kit and the Cy5 fluorophore (Invitrogen). A sample of DNA that has not been enriched by immunoprecipitation was subjected to LM-PCR and labeled with Cy3 fluorophore. Both immunopreciptation-enriched and non-enriched pools of labeled DNA were hybridized to the mouse promoter array described below. Data were analyzed as described previously (Ren et al. 2000) and the *P*-value threshold used to select target promoters for further analyses was determined empirically by testing randomly selected targets by standard ChIP/Q-PCR. Based on these experiments, we used $P < 0.01$, since our estimated false-positive rate was <10% using this threshold. Functional categories were assigned using both GO (http://www.fatigo.org) and manual inspection using PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed).

*Mouse promoter microarray design*

The strategy adopted to design our mouse promoter microarray is similar to the one used for our human promoter array (Laganiere et al. 2005). Briefly, full-length cDNAs were extracted from Refseq and mammalian gene collection (MGC) databases and filtered to eliminate redundancy and incomplete cDNAs. Their transcription start sites (TSS) were then located using the University of California at Santa Cruz (UCSC) genome browser (Karolchik et al. 2003) and the sequence ranging from 800 base pairs (bp) upstream of to 200 bp downstream from the TSS was extracted using the UCSC database assemblage (May 2004) (Karolchik et al. 2003). Primer pairs were designed using the Primer3 algorithm (Rozen and Skaletsky 2000) and the specificity was tested in silico using the vPCR algorithm (Lexa et al. 2001). When the primer pair gave no satisfactory vPCR results, a new primer pair was designed using primer3 and tested again. The process was iterated three times to generate primer pairs predicted to be efficient to amplify promoter regions from mouse genomic DNA for almost all of our selected genes. This strategy was adopted after preliminary results showed that a more simple primer design approach did not generate good results when we tried to amplify promoter regions from mouse genomic DNA. This primer design pipeline allows us to design primer pairs to amplify promoter regions from mouse genomic DNA with a success rate of ~80%. At the date of the download (November 2004) 17,947 RefSeq and 16,390 MGC entries were retrieved after the filtering process, and 18,892 of them were selected and submitted to primer design. Finally, primers were obtained for 18,655 promoters, whose identities will be provided on request.

*Statistical analysis*

Numbers of samples for each group used for the experiments are at least three or are indicated in the figure legends. Values were presented as mean ± standard error. An unpaired two-tailed Student's *t*-test or the log-rank version of the Kaplan-Meier survival test was used to calculate *P* values.

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