0888-8809/05/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 19(10):2466–2477
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doi: 10.1210/me.2004-0529

A Nuclear Receptor Atlas: Macrophage Activation

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Macrophage activation is an essential cellular process underlying innate immunity, enabling the body to combat bacteria and other pathogens. In addition to host defense, activated macrophages play a central role in atherogenesis, autoimmunity, and a variety of inflammatory diseases. As members of the Nuclear Receptor Signaling Atlas (NURSA) program, we employed quantitative real-time PCR (qPCR) to provide a comprehensive assessment of changes in expression of the 49 members of the murine nuclear receptor superfamily. In this study, we have identified a network of 28 nuclear receptors associated with the activation of bone marrow-derived macrophages by lipopolysaccharide or the prototypic cytokine interferon γ. More than half of this network is deployed in three intricate and highly scripted temporal phases that are unique for each activator. Thus, early receptors whose expression peaks within 4 h after lipopolysaccharide exposure, such as glucocorticoid receptor, peroxisome proliferator-activated receptor γ, and neuronal growth factor 1B, are found as late rising markers of the interferon γ cascade, occurring 16 h or later. The discovery of precise serial expression patterns reveals that macrophage activation is the product of an underlying process that impacts the genome within minutes and identifies a collection of new therapeutic targets for controlling inflammation by disruption of presumptive regulatory cascades. (*Molecular Endocrinology* 19: 2466–2477, 2005)

MACROPHAGES ARE THE major differentiated cell type of the mononuclear phagocyte system and serve as key effectors in antimicrobial defense, atherogenesis, autoimmunity, and other inflammatory diseases (1). By nature, they exist in a benign state but are able to sense and respond to microbial products and cytokines to mount an inflammatory response to eliminate offending pathogens. Such stimuli arm the macrophage by activating their capacity to phagocytose, process, and present antigens, and elaborate inflammatory mediators. Numerous extracellular inducers of macrophage activation have been identified, among the most studied of which are lipopolysaccharide (LPS) and interferon γ (IFN-γ). The molecular nature of activation, however, is largely not understood. The goal of this work is to use the nuclear receptors as a prototypic regulatory family to formulate a strategy to address this problem.

LPS is a structurally heterogeneous material contained within the cell wall of gram-negative bacteria and is recognized by animals as a molecular correlate to infection. It binds to Toll-like receptor 4, triggering multiple signaling cascades including those mediated through the transcription factor nuclear factor (NF)-κB and the Janus N-terminal kinase and p38 kinase pathways (2). LPS elicits multiple macrophage pro- and antiinflammatory cytokines, and the resulting effects may be protective or deleterious. Pretreatment of animals with LPS protects against bacterial infection and animals with mutations in the LPS receptor have enhanced pathogen susceptibility (3–7), yet LPS is one of the main causes of shock in sepsis. Hence, localized LPS responses triggered by small inoculums of gram-negative bacteria may be beneficial, but large doses that trigger systemic effects may be fatal.

First Published Online July 28, 2005

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Abbreviations: COUP-TF, Chicken ovalbumin upstream promoter-transcription factor; ER, estrogen receptor; ERR, estrogen-related receptor; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; GR, glucocorticoid receptor; LPS, lipopolysaccharide; LXR, liver X receptor; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; MR, mineralocorticoid receptor; MSF, macrophage serum-free; NF, nuclear factor; NGFIB, neuronal growth factor 1B; NOR, neuron-derived orphan receptor; NURR, nuclear receptor-related; NURSA, Nuclear Receptor Signaling Atlas; PPARγ, peroxisome proliferator-activated receptor γ; qPCR, quantitative real-time PCR; RAR, retinoic acid receptor; ROR, RAR-related orphan receptor; RXR, retinoid X receptor; TR, thyroid receptor; VDR, vitamin D receptor.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

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IFN-γ is a cytokine secreted by activated T cells and natural killer cells. Binding to its cognate receptor results in the activation of Janus kinase-signal transducer and activator of transcription and other signal transduction pathways involved in macrophage activation. IFN-γ-induced signals alter macrophage functions in immune surveillance, tumor suppression, and antiproliferative and antimicrobial responses and elicit a distinct set of inflammatory mediators (8).

Modification of gene expression is the mechanistic foundation for stimulus-induced activation of macrophages (1). Members of the NF-κB, signal transducer and activator of transcription, activator protein-1, CCAAT enhancer-binding protein, and interferon regulatory factor families are well-described transcription factors controlling macrophage gene expression. Whereas the glucocorticoid receptor (GR) has long been used as a potent antiinflammatory drug target, a growing body of work has implicated other members of the nuclear receptor superfamily, including the peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs), in the transcriptional control of macrophage lipid homeostasis and inflammation. Given the importance of this limited number of nuclear receptors in controlling macrophage function, we sought to identify the full complement of nuclear receptors expressed within the macrophage lineage. Twenty-eight receptors, several of which were not previously known to be expressed in the macrophage, were identified, and their temporal patterns of expression suggest transcriptional cascades involving multiple ligand-responsive receptors. This study provides a wealth of information to be exploited in understanding the dynamics of the activation process. Moreover, it demonstrates the value of comprehensive expression profiling of a regulatory gene family to provide hypothesis-driven approaches for dissecting a complex biological process.

RESULTS AND DISCUSSION

Mouse primary bone marrow-derived macrophages were exposed to media containing LPS or IFN-γ, and the resulting expression of inflammatory target genes and the 49 nuclear receptors were assessed over a 24-h time course using a high-throughput qPCR platform. RNA was isolated from cells harvested at 0, 0.5, 1, 2, 4, 8, 16, and 24 h after stimulation (Fig. 1) along with a parallel set of control samples from nonstimulated cells.

The Atlas of Macrophage Nuclear Receptors

Composite gene expression analysis of the superfamily at all time points revealed the presence of 28 of the 49 known nuclear receptors in macrophages. These include nine members of the endocrine receptor family, which are activated by high-affinity hormonal lipids, six adopted heterodimeric orphan receptors, which are regulated by low-affinity dietary lipids, and 13 true orphan receptors, including seven constitutive activators and six constitutive repressors (Fig. 2A) (9). Figure 2B shows a complete tabulation of the expressed and nonexpressed receptors along with their classification and nomenclature. Interestingly, primary bone marrow-derived (Fig. 2, A and B) and RAW 264.7 (data not shown) macrophages express the identical set of 28 nuclear receptors, which suggest this collection may serve as a molecular signature of the macrophage. Indeed, analysis of 39 other tissues from the body shows that this signature is unique (Bookout, A. L., M. Downes, R. T. Yu, R. M. Evans, and D. J. Mangelsdorf, unpublished data). LPS or IFN-γ stimulation resulted in dynamic and unanticipated patterns of receptor transcription in bone marrow-derived macrophages. Primary macrophages were also exposed to fresh media without LPS or IFN-γ and assessed for changes in nuclear receptor, cytokine, and chemokine expres-

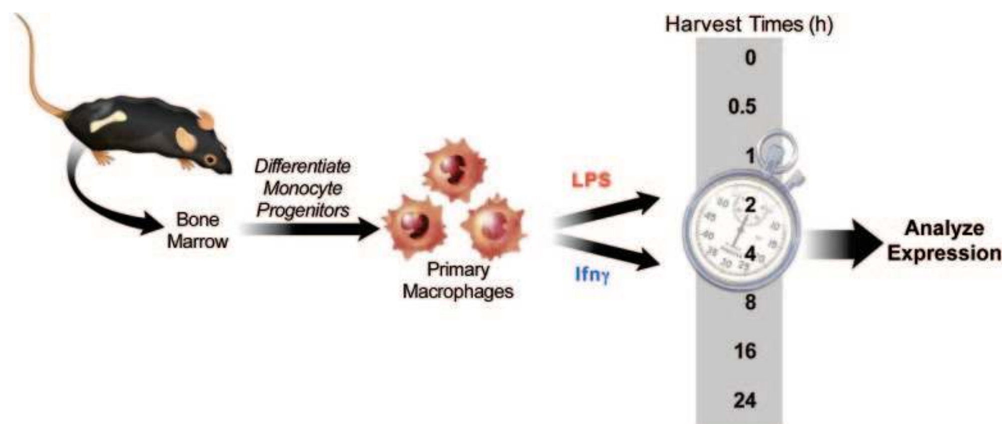

Fig. 1. Experimental Design
Monocyte progenitors from C57BL6/J mice were obtained and differentiated into primary macrophages. Primary macrophages were exposed to LPS or IFN-γ and harvested at the indicated time points. Samples were processed and subjected to quantitative PCR analysis.

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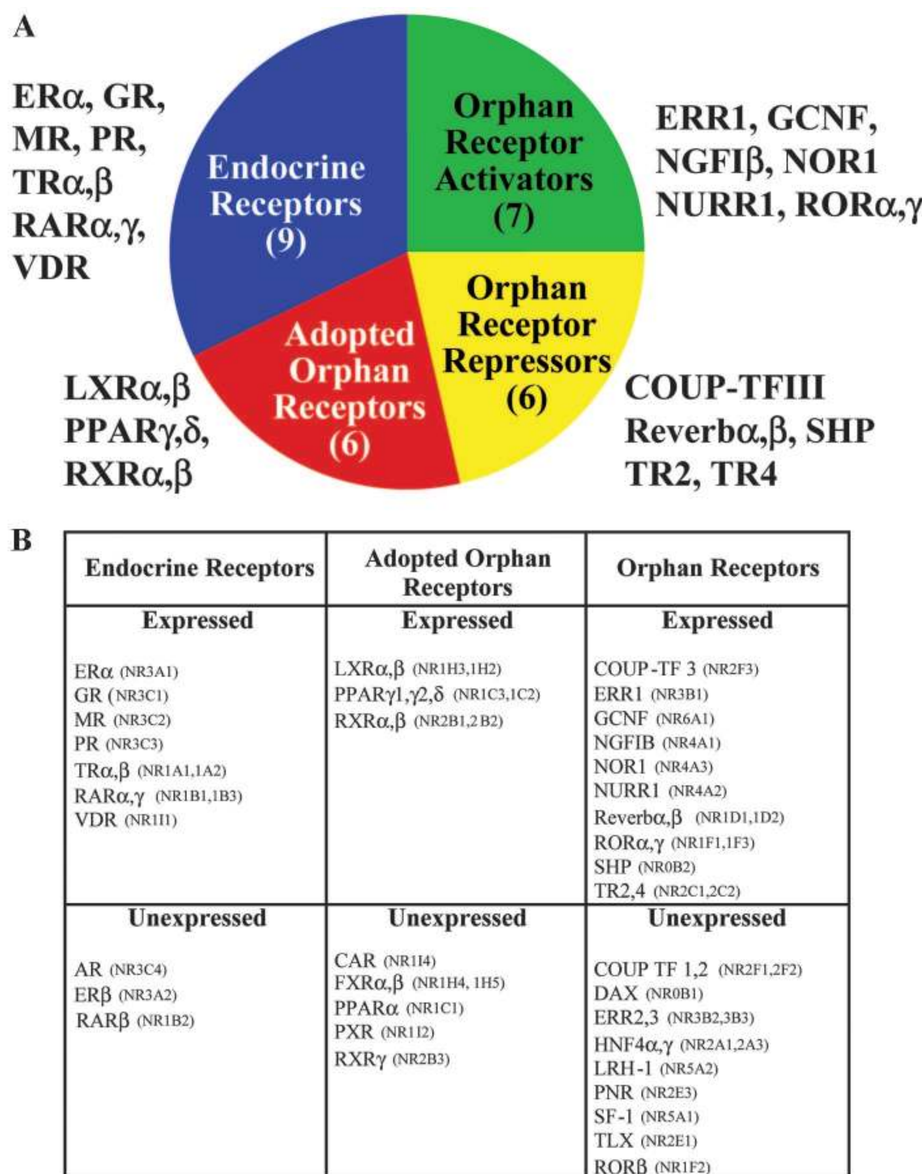

Endocrine Receptors	Adopted Orphan Receptors	Orphan Receptors
Expressed	**Expressed**	**Expressed**
ERα (NR3A1)	LXRα,β (NR1H3,1H2)	COUP-TF 3 (NR2F3)
GR (NR3C1)	PPARγ1,γ2,δ (NR1C3,1C2)	ERR1 (NR3B1)
MR (NR3C2)	RXRα,β (NR2B1,2B2)	GCNF (NR6A1)
PR (NR3C3)		NGFIB (NR4A1)
TRα,β (NR1A1,1A2)		NOR1 (NR4A3)
RARα,γ (NR1B1,1B3)		NURR1 (NR4A2)
VDR (NR1I1)		Reverbα,β (NR1D1,1D2)
		RORα,γ (NR1F1,1F3)
		SHP (NR0B2)
		TR2,4 (NR2C1,2C2)
Unexpressed	**Unexpressed**	**Unexpressed**
AR (NR3C4)	CAR (NR1I4)	COUP TF 1,2 (NR2F1,2F2)
ERβ (NR3A2)	FXRα,β (NR1H4, 1H5)	DAX (NR0B1)
RARβ (NR1B2)	PPARα (NR1C1)	ERR2,3 (NR3B2,3B3)
	PXR (NR1I2)	HNF4α,γ (NR2A1,2A3)
	RXRγ (NR2B3)	LRH-1 (NR5A2)
		PNR (NR2E3)
		SF-1 (NR5A1)
		TLX (NR2E1)
		RORβ (NR1F2)

Fig. 2. The Composition of Nuclear Receptors Expressed in Macrophages

A, Twenty-eight of 49 known nuclear receptors are expressed in the macrophage. These include nine endocrine receptors, six adopted orphan receptors that bind to low-affinity dietary lipids, and 13 orphan receptors, consisting of seven constitutive activators and six constitutive repressors. Constituent receptors of each of these classes are listed. B, Tabular listing of nuclear receptors expressed or nonexpressed in macrophages with their unified nomenclature system names listed in *parentheses* (77). Receptors were deemed unexpressed if cycle threshold (Ct) values exceeded 35. HNF, Hepatocyte nuclear factor; SF, steroidogenic factor; CAR, constitutive androstane receptor; FXR, farnesoid X receptor; PXR, pregnane X receptor; AR, androgen receptor.

sion. In most cases, there were no changes in expression over the entire time period. Several transcript profiles for stimulated primary bone marrow-derived macrophages are discussed below, and the entire expressed data set is available in Supplemental Fig. 3 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org and on the Nuclear Receptor Signaling ATLAS (NURSA) web site at www.nursa.org.

The Macrophage Endocrine Receptor Family

As depicted in Fig. 2, nine of the 12 members of the endocrine receptor family are expressed within the macrophage, including the GR, mineralocorticoid receptor (MR), estrogen receptor α (ERα), progesterone receptor (PR), vitamin D receptor (VDR), thyroid receptors α and β (TRα,β), and retinoic acid receptors α and γ (RARα,γ).

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The GR and MR are highly related and bind identical DNA response elements. In addition, the MR binds to both mineralocorticoids and glucocorticoids with high affinity (10). Despite their proclivities for common ligands and DNA binding sites, MR and GR direct distinct, virtually complementary transcriptional programs. The precise mechanisms conferring their specificity are unknown, although the conversion of glucocorticoids to inactive metabolites by 11β-hydroxysteroid dehydrogenase enzyme 2 is suggested as an important mechanism to inactivate intracellular glucocorticoids in aldosterone target tissues (11). Interestingly, 11β-hydroxysteroid dehydrogenase enzyme 2 is not expressed in resting or stimulated macrophages (data not shown). Given the absence of this ligand-deactivating enzyme and the fact that glucocorticoids are approximately 100-fold more concentrated than mineralocorticoids in the serum, it is probable that circulating glucocorticoids activate the MR in the macrophage. Upon LPS stimulation, GR and MR are divergently regulated, because the GR is induced by approximately 5-fold over baseline, whereas the MR is reciprocally and dramatically suppressed within 4 h (Fig. 3A). The function of MR in the macrophage is unknown, although mineralocorticoids have been shown to increase macrophage oxidative stress and recent studies suggest that the highly specific MR antagonist eplerenone is antiinflammatory (12–15). By contrast, GR and its agonists have an established antiinflammatory role in the macrophage by virtue of its ligand-dependent inhibition of Janus N-terminal kinase, MAPK activation and the proinflammatory transcription factor complexes activator protein-1 and NF-κB (16–22). It is tempting to speculate that the opposing regulation of GR and MR upon LPS stimulation could represent a form of inflammatory modulation during the first 24 h of macrophage activation. Such effects could contribute to the phenomenon of endotoxin tolerance, in which a primary challenge with LPS results in insensitivity to a secondary exposure (2). In contrast to LPS, IFN-γ shows an approximately 4-fold induction of both GR and MR with no evident attenuation (Fig. 3B). This leads to the interesting possibility that the antiinflammatory effects of glucocorticoids and mineralocorticoid antagonists may differ depending upon the nature of the activating stimulus.

Estrogen and progesterone are well recognized for their effects on reproductive tissues, whereas vitamin D is known for its involvement in calcium and phosphorus metabolism. Interestingly, however, these high-affinity nuclear receptor ligands, like glucocorticoids, modulate inflammation. The multiple sclerosis-like experimental autoimmune encephalomyelitis mouse model is attenuated by estrogen, and estrogen and progesterone inhibit LPS and IFN-γ-elicited inflammatory cytokines (23–29). Vitamin D likewise suppresses autoimmune diseases including autoimmune encephalomyelitis, and the VDR is required to develop experimental asthma (30–35). Consistent with other reports, qPCR analysis identified ERα, PR, and VDR, but not ERβ, in macrophages (Fig. 2B) (23, 36–38). Exposure to LPS decreases the expression of ERα and PR by up to 70% and 85%, respectively, whereas IFN-γ treatment results in a 10-fold increase in ERα and more than a 3-fold increase in PR transcript levels (Fig. 3, A and B; and Supplemental Fig. 3). Alternatively, LPS enhances the expression of VDR by nearly 6-fold at 24 h, and IFN-γ stimulates VDR transcription by more than 20-fold just 4 h after treatment (Fig. 3, A and B). Again, these profiles lead to testable predictions as to when activated macrophages may become sensitive to vitamin D_3, estrogenic, or progesterogenic modulation.

TRα and TRβ are important regulators of metabolic rate and oxidative metabolism, but little is known about their functions in macrophages, where both are expressed (Fig. 2, A and B). Interestingly, TRβ is dramatically induced by more than 10-fold with IFN-γ but

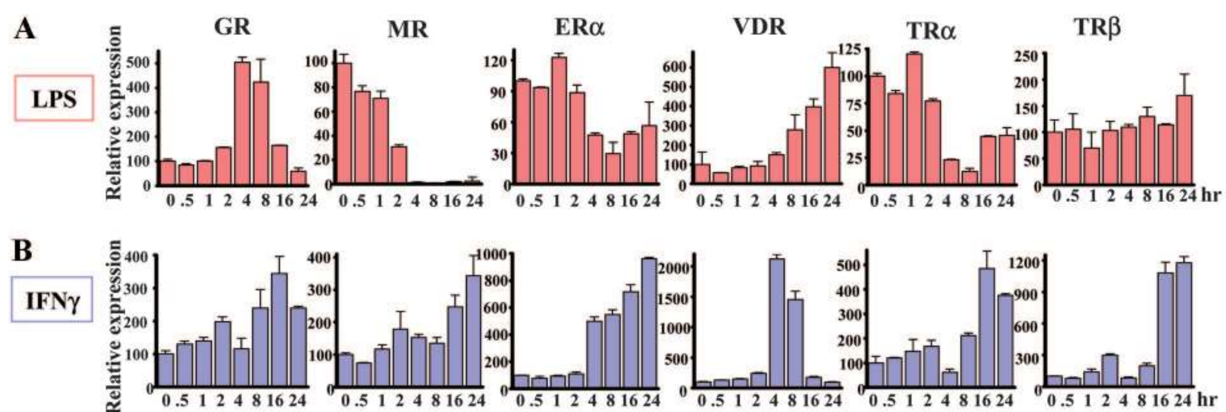

Fig. 3. Stimulated Expression Profiles of Endocrine Nuclear Receptors

A, LPS induces the expression of GR and VDR but inhibits the expression of MR, ERα, and TRα. LPS does not alter TRβ mRNA levels. B, IFN-γ enhances the expression of each of the aforementioned receptors. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points show expression relative to time zero. *Error bars* represent SD.

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unaffected by LPS treatment (Fig. 3, A and B). In contrast, TRα levels are suppressed to less than one fourth its initial levels within 4 h of LPS exposure, whereas it is induced by almost 5-fold with IFN-γ (Fig. 3, A and B). Because IFN-γ is known to induce the macrophage respiratory burst *in vitro* (39), it is possible that up-regulation of TRs could help to fuel enhanced macrophage oxidative metabolism.

The Macrophage Adopted Orphan Receptor Family

Members of the adopted orphan family heterodimerize with retinoid X receptors (RXR), and function as sensors for fatty acids (PPARs), oxysterols (LXRs), bile acids (farnesoid X receptors), and xenobiotics (constitutive androstane receptor and steroid and xenobiotic receptor) (9). Together, they regulate diverse aspects of lipid metabolism, storage, and transport. Notably, members of the PPAR and LXR subfamilies have also emerged as important regulators of inflammation. Six of 12 members of the adopted orphan receptor family are expressed within the macrophage, including PPARγ1/2 and δ, LXRα and β, and RXRα and β (Fig. 2, A and B).

PPARγ mediates the insulin-sensitizing effects of the thiazolidinedione class of drugs for diabetes mellitus and plays a critical role in adipocyte differentiation and lipid storage. Its two receptor isoforms, PPARγ1 and PPARγ2, are expressed in the macrophage, where they regulate cholesterol uptake and efflux (40, 41). Synthetic PPARγ agonists inhibit LPS and IFN-γ-elicited inflammatory mediators including TNF-α, IL-1β, IL-6, matrix metalloproteinase-9, and inducible nitric oxide synthase (iNOS), although some of these effects may not be dependent upon PPARγ (42–45). In macrophages exposed to LPS, PPARγ1 and PPARγ2 expression are dramatically suppressed within 2 h and relative mRNA levels remain low for the duration of the analysis, whereas IFN-γ results in modest 1.5- to 3-fold inductions (Fig. 4, A and B; and Supplemental Fig. 3). Thus, by 24 h there is an approximately 40-fold difference in PPARγ1 or 30-fold difference in PPARγ2 transcript levels between IFN-γ- and LPS-treated cells. Based on these results, it is predicted, for instance, that PPARγ agonists may have little impact on the expression of PPARγ target genes after LPS stimulation, due to low abundance of this receptor. Corroborating this, the high-affinity PPARγ agonist rosiglitazone fails to induce the established macrophage PPARγ targets ABCG1, CD36, and ADRP after 24 h of LPS cotreatment (Supplemental Fig. 1) (45).

PPARδ regulates genes involved in fatty acid catabolism and thermogenesis in fat and skeletal muscle and modulates inflammation in the skin and macrophage (46, 47). Treatment of macrophages with either LPS or IFN-γ results in approximately 4-fold induction of PPARδ expression at 16–24 h after exposure (Fig. 4, A and B). Unliganded PPARδ is thought to sequester inflammatory suppressor proteins (47), so its continued expression supports a potential modulatory site for both pathways even at late stages of activation.

LXRs are sterol-activated cholesterol sensors that regulate a battery of genes involved in cholesterol efflux, bile acid production, fatty acid synthesis, and lipid transport. Both LXRα and LXRβ are found in the macrophage, where they drive cholesterol efflux through their regulation of ATP binding cassette trans-

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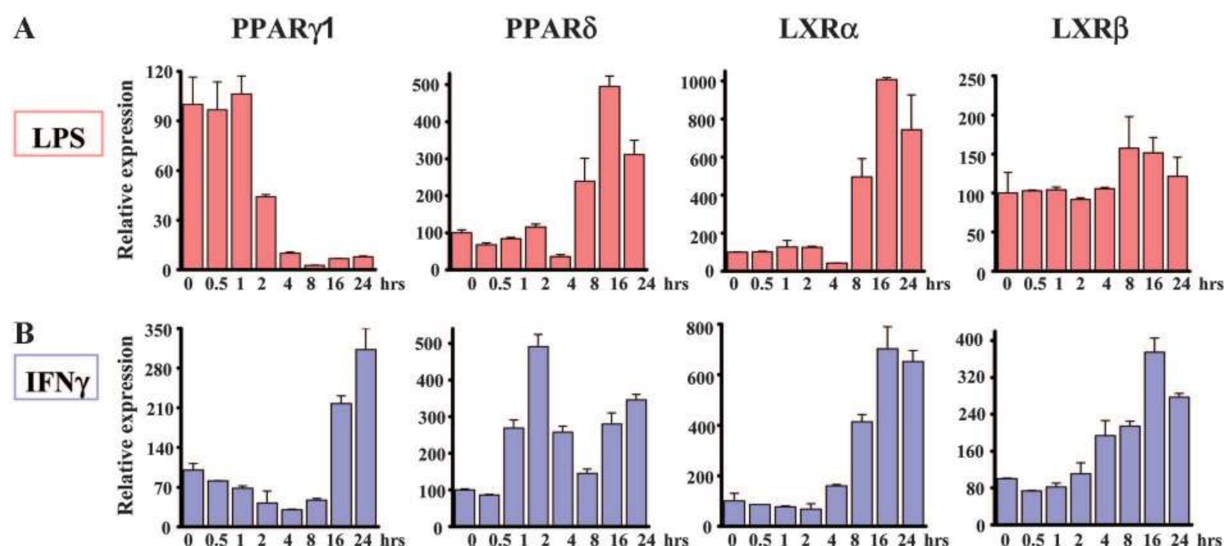

Fig. 4. Stimulated Expression Profiles of PPARs and LXRs

A, LPS enhances the expression of PPARδ and LXRα but inhibits PPARγ mRNA levels. LPS has minimal effects on LXRβ expression. B, IFN-γ stimulates the expression of each of the PPAR and LXR isoforms expressed within the macrophage. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points show expression relative to time zero. *Error bars* represent SD.

port proteins ABCA1 and ABCG1 (Fig. 2, A and B) (48). Furthermore, LXR synthetic agonists inhibit LPS-induced macrophage inflammatory signals including iNOS and cyclooxygenase 2 (49). After exposure to LPS, LXRα expression is induced by 10-fold at 16 h, whereas LXRβ levels are essentially unchanged (Fig. 4A). IFN-γ treatment enhances LXRα expression by approximately 8-fold and LXRβ expression by over 3-fold at 16 h (Fig. 4B). Notably, the more dynamic LXRα, but not LXRβ, has been identified as an important effector of innate antilisterial immunity, and LXRα expression is likewise enhanced by listeria infection (50). These results support the idea that LPS- and IFN-γ-induced pathways could, as with PPARδ ligands, be effectively targeted by LXR agonists (49).

The Orphan Nuclear Receptor Family

As shown in Fig. 2B, 13 of 25 members of the orphan nuclear receptor family are expressed in macrophages. These include both constitutive activators [neuronal growth factor 1B (NGFIB), neuron-derived orphan A receptor (NOR) 1, nuclear receptor-related (NURR) 1, estrogen-related receptor (ERR) 1, RAR-related orphan receptor (ROR) α/γ, and germ cell nuclear factor] and constitutive repressors [chicken ovalbumin upstream promoter-transcription factor (COUP-TF) 3, orphan receptor encoded on the noncoding strand of the thyroid receptor-α and -β, small heterodimeric partner, TR2, and TR4] that bind DNA either as monomers, homodimers, or RXR heterodimers. Despite such significant representation, their functions within the macrophage are virtually unknown.

Among the orphan receptors expressed in macrophages, the NR4A subgroup including NGFIB, NURR1, and NOR1 (Fig. 2, A and B) shows an unusual nested expression pattern. The crystal structure or NURR1 reveals no ligand binding pocket, indicating that its constitutive activity, if modulated, would be through a second message signaling pathway (51). These receptors mediate immediate-early responses to numerous stimuli acting at the cell surface, bind to diverse promoter elements as monomers, homodimers, or as heterodimers (except NOR1) with RXR, and may be antagonized by glucocorticoids at certain promoters (52). Activation of NGFIB has been reported to promote apoptosis in a caspase-independent manner in macrophages, to interact with Bcl-2 during translocation to mitochondria, and to mediate negative selection of T cells and other cells during development. Additionally, NGFIB opposes the proinflammatory signaling molecule NFκB in Jurkat cells and TNF-α-mediated apoptosis in mouse embryonic fibroblasts (53–56). In response to extracellular signals, NR4A receptors may function as molecular switches between transcriptional programs of activation, differentiation, or self-destruction via dynamic interactions with NFκB and glucocorticoid signaling (57, 58). An unexpected expression cascade was discovered in LPS-treated macrophages, initiated by a 15-fold induction of NGFIB within 1 h. This is followed by an approximate 1000-fold induction of NOR1 in 2–4 h, during which NGFIB returns to basal levels. A final wave of induction is seen with Nurr1 rising 24-fold by 16 h, during which Nor1 returns to its initial levels (Fig. 5A). This sequential pattern of expression is consistent with a transcriptional cascade, although such a possibility remains speculative (59). Consistent with this serial regulation hypothesis, an isoform of Nurr1 can down-regulate the transcriptional activity of NGFIB, which could complement the transcriptional silencing of NGFIB expression observed after 4 h of exposure to

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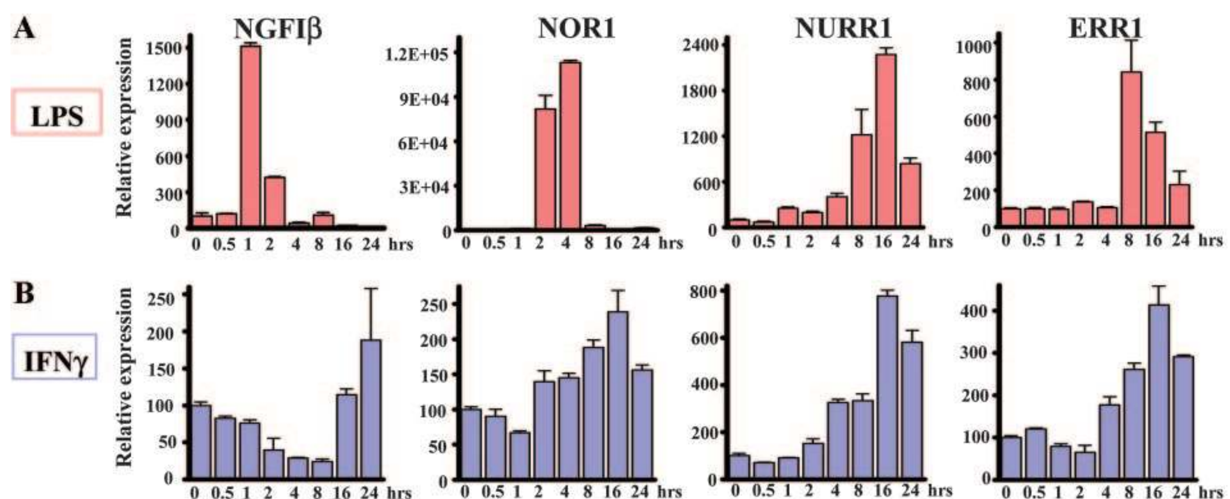

Fig. 5. Stimulated Expression Profiles of the NR4A Subgroup and ERR1

A, LPS stimulation induces robust and transient expression of NGF1B, NOR1, NURR1, and ERR1. B, IFN-γ stimulation causes modest stimulation of NGF1B, NOR1, NURR1, and ERR1 transcription. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points show expression relative to time zero. *Error bars* represent SD.

LPS (Fig. 5A) (60). In contrast, IFN-γ treatment enhances the expression of all of the NR4A receptors in a parallel rather than sequential manner with transcription peaking 2- to 8-fold at 16–24 h after exposure (Fig. 5B).

ERR1 is a constitutively active orphan receptor and is suggested to be a primary effector of the tissue-specific coactivator PGC-1 and its transcriptional program to drive oxidative phosphorylation and mitochondrial biogenesis (61, 62). Consistent with a previous report, we identified ERR1 expression in macrophages, where its function is unknown (Fig. 2, A and B) (63). Interestingly, LPS induced ERR1 expression more than 8-fold at 8 h after exposure, followed by a decline of levels to 2-fold above baseline at 24 h (Fig. 5A). IFN-γ likewise enhanced ERR1 expression, with peak levels up more than 4-fold at 16 h after treatment (Fig. 5B). If indeed ERR1 proves to have a role in macrophage inflammatory responses, it too could be a pharmacological target. Although no agonist for this receptor has been identified, multiple antagonists including diethylstilbestrol and the organic pesticides toxaphene and chlordane have been described, suggesting that therapeutic modulation of ERR1 could be possible (64, 65). It is also noteworthy that ERR1 joins a panel of receptors identified in these studies, including TRα, TRβ, and PPARδ that are each involved in oxidative metabolism and are expressed in macrophages, in which the role for oxidative metabolism remains poorly understood.

LPS and IFN-γ Induce Distinct but Overlapping Inflammatory Changes

To further explore the nature of macrophage activation and to validate our LPS and IFN-γ stimulations, we assessed the temporal expression of macrophage inflammatory mediators elicited by either agent. Monocyte chemoattractant protein-1 (MCP-1) binds to chemokine receptors and recruits monocytes to areas of inflammation (66–68), whereas iNOS catalyzes the formation of nitric oxide, an oxidative mediator capable of destroying microbes or damaging host tissues. Previous reports demonstrate that both MCP-1 and iNOS are induced with either LPS or IFN-γ (45, 69, 70). In primary macrophages stimulated with LPS, MCP-1 transcription is induced within 1 h, peaks at more than 70 times prestimulated levels within 2 h, and diminishes to nearly basal levels at 24 h (Fig. 6A). In contrast, IFN-γ exposure causes a more graded induction of MCP-1, peaking at 8 h and only partially attenuating at 24 h (Fig. 6B). iNOS expression increases a remarkable 3500-fold over its basal levels 8 h after LPS stimulation and is similarly induced by nearly 800-fold after IFN-γ exposure (Fig. 6, A and B). Macrophage inflammatory protein-2 (MIP-2) is a macrophage-derived, proinflammatory chemokine involved in neutrophil chemotaxis to sites of injury or inflammation. Consistent with other studies, LPS and IFN-γ differentially regulate MIP-2 expression; it is enhanced almost 100-fold by LPS but suppressed to 20% of its basal levels by IFN-γ (Fig. 6, A and B) (71, 72). The proinflammatory cytokines IL-1β, IL-6, and TNFα are critical mediators of the acute phase response, and each is potently induced after LPS treatment (2). IL-6 transcript rises an astounding 90,000-fold within 4 h after exposure to LPS, yet shows an equally precipitous fall in expression over the next 4–8 h (Fig. 6A). Inductions of TNFα and IL-1β message are more sustained, peaking 12-fold at 2 h and over 6000-fold at 8 h, respectively, after LPS exposure but remaining elevated for the duration of the experiment. IFN-γ triggers a similar, albeit more modest induction profile for IL-6 with a peak expression of approximately 70-fold over baseline occurring 8 h after treatment (Fig. 6B). In contrast, TNFα levels are relatively unaffected and IL-1β levels are transiently suppressed by IFN-γ treatment (Fig. 6B). These data represent a selected fraction of the changes in gene expression attendant with macrophage activation but fortify the concept that macrophage activation

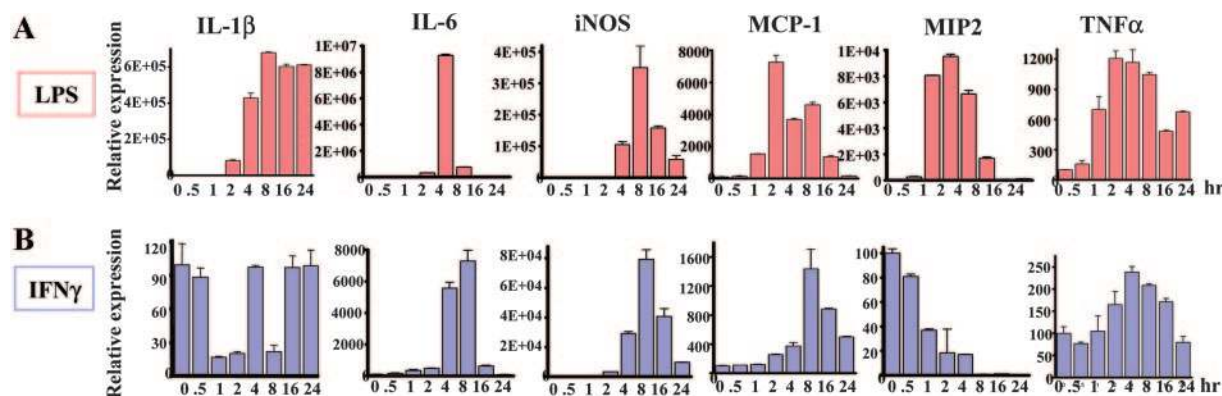

Fig. 6. LPS and IFN-γ Variably Alter the Expression of Inflammatory Mediators

A, LPS induces the expression of IL-1β, IL-6, iNOS, MCP-1, MIP2, and TNFα. B, IFN-γ enhances transcription of IL-6, iNOS, and MCP-1 but suppresses MIP-2 and IL-1β and has minimal effects on TNFα. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points show expression relative to time zero. *Error bars* represent SD.

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encompasses a quantifiable and temporally dynamic process.

Conclusion

We have determined the entire complement of nuclear receptors associated with macrophage activation by LPS and IFN-γ. Using quantitative PCR, we have identified 28 nuclear receptors that appear to participate in a process that is more dynamic and complex than previously presumed. One of the most unusual features of our analysis is the ephemeral expression of so many receptors (such as ERα, ERR1, NGF1β, NOR1, NURR1, PPARδ, TRβ, and VDR) whose expression appears to demarcate steps on the way to macrophage activation (Fig. 7). Minimally, this serial expression pattern reveals that macrophage activation is the product of an underlying process that impacts the genome within minutes and progresses through a continuum for the next 24 h. In the various expression profiles presented here, it is evident that LPS and IFN-γ elicit similar yet distinct or in some cases opposing cytokine and nuclear receptor transcriptional responses. This is particularly interesting given the mutual capacity of IFN-γ or LPS to prime macrophage responses to the other signal (73–76).

How can one try to determine whether these changing patterns of gene expression are important? This is a key question and one that has been addressed in other systems, such as *Drosophila* differentiation, where it is clear that a self-governing transcriptional cascade produces a unique output, namely the anterior-posterior axis. In this example, a key component in the process is defined by the time and location when a gene product reaches its maximum concentration. By analogy, our ability to accurately quantify changes in nuclear receptor mRNA offers a level of analysis not amenable through standard DNA microarray technology. One feature from the study is the striking responsiveness of certain receptors such as the NR4A subgroup and ERR1 or PPARδ and VDR, which show dramatic inductions after LPS or IFN-γ treatment, respectively, indicating rapid effects at the promoters of these genes (Fig. 7). A search for common upstream response elements would identify the potential triggers of each activation step. A second feature is the impressive decline of receptor mRNA levels, indicating likely repression of the receptor genes themselves, coupled with extremely short mRNA half-lives. This raises the question as to the existence of decay sequences that direct these processes and raises a parallel question as to the half-lives of the encoded proteins. Even without this knowledge, we can presume that peak protein levels of a specific receptor will be at least roughly guided by its peak mRNA concentration, and protein immunoblot analysis of selected receptors after stimulation, including GR and ERR1, fortify this contention (Supplemental Fig. 2). Juxtaposition of different receptor expression patterns after LPS or IFN-γ treatment (Fig. 7) supports the concept of a combinatorial regulatory code comprised of a succession of transcriptional activators and repressors that trace back to the zero time point of the experiment. Broadly, nuclear receptor expression changes are early, intermediate, or late markers of macrophage activation (Fig. 7). This triphasic classification predicts potential cooperative effects of ligand combinations in inhibiting inflammatory responses. Some receptors in these temporal cascades are expected to be key in propelling the cell through the process of activation, whereas others may play a more cryptic role in this process. This former group could be particularly important for effecting functional properties associated with macrophage activation such as MCP-1, iNOS, MIP2, IL-1, IL-6, and TNFα expression. Furthermore, the use of knockout or knockdown approaches such as RNA interference could be valuable in determining the extent to which intermediary or ephemerally expressed receptors contribute to downstream expression cascades and activation events.

Little is known about transcriptional programs controlled by nuclear receptors within the macrophage. Their regulation is made more complex by interactions with corepressors or coactivators and their allosteric modification by ligands. Further work to define the constellation of coregulators and endogenous macrophage ligands is already underway. Surprisingly, 18 receptors described in this study respond to known ligands, expanding the macrophage as a potential regulatory target. Coupled with elucidation of the complete atlas of macrophage nuclear receptors shown here, further studies promise to functionally dissect the inflammatory response and may highlight new therapeutic approaches to infectious or inflammatory diseases.

MATERIALS AND METHODS

Derivation of Differentiated Bone Marrow Macrophages

Marrow was flushed from the femur and tibia of wild-type male C57Bl6/J animals (The Jackson Laboratory, Bar Harbor, ME), purified through a Ficoll-Paque gradient (Amersham Biosciences, Arlington Heights, IL), and cultured in DMEM containing 20% endotoxin-reduced fetal bovine serum (Sigma, St. Louis, MO) and 30% L929 conditioned medium for 5 d.

LPS and IFN-γ Stimulations

Differentiated macrophages were counted and replated 12 h before stimulation using 4×10^6 cells per 6-cm plate and incubated in macrophage serum-free (MSF) media (Invitrogen, Carlsbad, CA). For time course studies, MSF media were replaced at the initiation of the experiment with fresh MSF media (for time point of zero and control cells) or fresh MSF containing *Escherichia coli* 026:B6-derived LPS 1 μg/ml (Sigma) or mouse IFN-γ 2 ng/ml (BD Pharmingen, San Diego, CA). Cells were maintained at 37 C in a 7% CO_2 incubator over the experimental time course.

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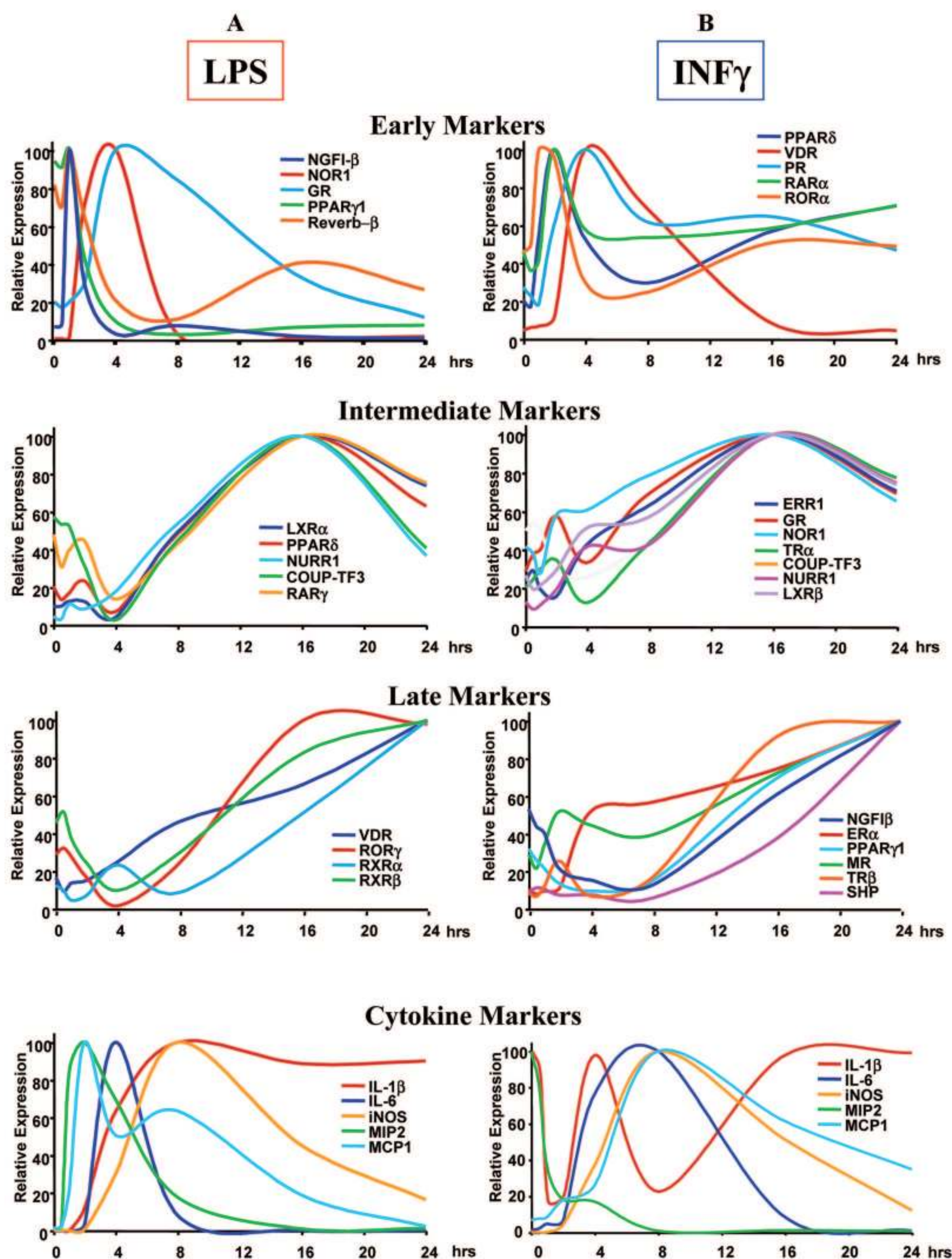

Fig. 7. Macrophage Nuclear Receptor Expression Cascades

A, LPS exposure results in rapid, sequential, and transient expression cascades of nuclear receptors. These can be divided into early altered genes (expression peaking within 4 h) including NGFIβ, NOR1, GR, PPARγ, and ReVerbβ, intermediate markers (expression peaking within 16 h) including LXRα, PPARδ, Nurr1, COUP-TF3, and RARγ, and late altered genes (expression peaking or still increasing at 24 h) such as VDR, RORγ, RXRα, and RXRβ. The inflammatory markers IL-1β, IL-6, iNOS, MIP2, and MCP1 are variably induced by LPS between 0 and 24 h. B, IFN-γ stimulation induces distinct cascades of nuclear receptor expression. Early markers including PPARδ, VDR, PR, RARα, and RORα have peak expressions within 4 h. Intermediate markers are induced within 16 h, including ERR1, GR, Nor1, TRα, COUP-TF3, Nurr1, and LXRβ. Other receptor expression peaks occur at or later than 24 h after stimulation, including NGFIβ, ERα, PPARγ1, MR, TRβ, and small heterodimer partner (SHP), functioning as late markers. Inflammatory mediators including IL-6, iNOS, and MCP-1 are induced by IFN-γ, whereas MIP-2 is suppressed and IL-1β levels vacillate. The maximum expression detected over the entire experimental time course for each indicated receptor (normalized to 36B4) was assigned an expression value of 100. Relative expression at all time points of the experimental time course are depicted.

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qPCR Procedures

All treatments were performed in triplicate. For time course studies, cells were harvested at each of the indicated time points of 0, 0.5, 1, 2, 4, 8, 16, and 24 h. For ligand treatment studies (Supplemental Fig. 1), cells were exposed to LPS 1 μg/ml with or without rosiglitazone 1 μM in DMSO or DMSO vehicle control solution. Upon harvesting, media were aspirated and cells were lysed using 1 ml of Trizol reagent (Invitrogen). Total RNA was extracted from Trizol. First-strand cDNA was synthesized with 1 μg of purified RNA using SuperScript II and Random Primers (Invitrogen). Samples were subsequently treated with ribonuclease H (Invitrogen). A 384-well microtiter dish format was used for qPCRs with SYBR green (Sigma), and final reaction volumes were 10 μl. High-throughput processing was achieved using a semiautomated Beckman (Fullerton, CA) liquid handler, an ABI Prism 7900HT (Applied Biosystems, Foster City, CA), and sequence detection system software. For each biological sample, qPCRs were performed in quadruplicate and expression was normalized to 36B4 expression. Bar graphs represent the averaged relative expression of the triplicate biological samples and the SD, assigning the initial time point a relative expression of 100% for each indicated transcript.

Primer sequences for all nuclear receptors and inflammatory markers assessed in this study are available on the NURSA web site at www.nursa.org.

Immunoblot Protein Analysis

Bone marrow-derived macrophages were stimulated with LPS (as above), and total cell lysates were obtained at each of the indicated time points by adding 1 ml of sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 0.01% bromophenol blue, and protease inhibitors (Roche, Indianapolis, IN)]. Samples were separated on 10% SDS-PAGE gel and transferred to nitrocellulose (GE Osmonics, Minnetonka, MN). After confirming equal protein loading and transfer by staining with Ponceau S, blots were incubated in blocking solution (0.1% Tween in PBS) for 1 h. Blots were incubated for 1 h at 25 C with either GR primary antibody (MA1-510; Affinity BioReagents (Golden, CO)) diluted 1:500 or estrogen-related receptor α antibody (gift of Dr. Vincent Giguere, McGill University, Montreal, Quebec, Canada) 1:8000. Blots were washed three times in 0.1% Tween in PBS, incubated in secondary antibody conjugated to horseradish peroxidase, rewashed in 0.1% Tween in PBS, placed in ECL substrate (SuperSignal; Pierce, Rockford, IL), and exposed to film. Films were scanned and band densities were assessed using NIH ImageJ analysis software (Supplemental Fig. 2). The complete data set is available in Supplemental Fig. 3 and on the NURSA web site at http://www.nursa.org.

Experimental Animals

All animal experimentation described in this work was conducted in accordance with a Salk Institute approved Institutional Animal Use and Care Committee protocol.

Acknowledgments

We thank Helen Cho (The Salk Institute) for helpful discussions, Vincent Giguere (McGill University, Montreal, Quebec, Canada) for ERR1 antibody, Jackie Alvarez and the Salk Institute Q-PCR core facility for technical assistance, Jamie Simon for Fig. 1, and Lita Ong and Elaine Stevens for administrative support (all from The Salk Institute).

Received December 22, 2004. Accepted May 31, 2005.

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This work was funded through the support of the National Institutes of Health (NIH)/National Institute of Diabetes and Digestive and Kidney Diseases Nuclear Receptor Signaling Atlas orphan receptor program, Grant U19DK62434, and the Howard Hughes Medical Institute. G.D.B. is supported by the NIH Public Health Services Grant CA09370 and the University of California-San Diego Institute of Molecular Medicine. R.M.E. is an Investigator of the Howard Hughes Medical Institute at The Salk Institute for Biological Studies and March of Dimes Chair in Molecular and Developmental Biology.

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