Nuclear Receptors Nur77, Nurr1, and NOR-1 Expressed in Atherosclerotic Lesion Macrophages Reduce Lipid Loading and Inflammatory Responses

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Objective—Atherosclerosis is an inflammatory disease in which macrophage activation and lipid loading play a crucial role. In this study, we investigated expression and function of the NR4A nuclear receptor family, comprising Nur77 (NR4A1, TR3), Nurr1 (NR4A2), and NOR-1 (NR4A3) in human macrophages.

Methods and Results—Nur77, Nurr1, and NOR-1 are expressed in early and advanced human atherosclerotic lesion macrophages primarily in areas of plaque activation/progression as detected by in situ-hybridization and immunohistochemistry. Protein expression localizes to the nucleus. Primary and THP-1 macrophages transiently express NR4A-factors in response to lipopolysaccharide and tumor necrosis factor α. Lentiviral overexpression of Nur77, Nurr1, or NOR-1 reduces expression and production of interleukin (IL)-1β and IL-6 proinflammatory cytokines and IL-8, macrophage inflammatory protein-1α and -1β and monocyte chemoattractant protein-1 chemokines. In addition, NR4A-factors reduce oxidized–low-density lipoprotein uptake, consistent with downregulation of scavenger receptor-A, CD36, and CD11b macrophage marker genes. Knockdown of Nur77 or NOR-1 with gene-specific lentiviral short-hairpin RNAs resulted in enhanced cytokine and chemokine synthesis, increased lipid loading, and augmented CD11b expression, demonstrating endogenous NR4A-factors to inhibit macrophage activation, foam-cell formation, and differentiation.

Conclusion—NR4A-factors are expressed in human atherosclerotic lesion macrophages and reduce human macrophage lipid loading and inflammatory responses, providing further evidence for a protective role of NR4A-factors in atherogenesis. (Arterioscler Thromb Vasc Biol. 2006;26:2288-2294.)

Key Words: atherosclerosis ■ foam-cell formation ■ inflammation ■ macrophage function ■ nuclear orphan receptors ■ NR4A

Atherosclerosis is a chronic inflammatory disease involving deregulation of both the immune system and lipid metabolism.1,2 Macrophages, imperative in the innate immune system, are involved in the initiation, progression, and rupture of atherosclerotic lesions as well as in the initiation of smooth muscle cell (SMC)-rich pathologies like restenosis.3,4 At the onset of atherosclerosis, monocytes are locally recruited to the arterial vessel wall, where these cells differentiate into macrophages. These intimal macrophages ingest modified lipid particles and become lipid-laden foam cells that form a so-called fatty streak. In advanced atherosclerotic lesions, macrophages are localized primarily around a central lipid core and at the shoulder region of the plaque. At the latter site, which is known to be prone to rupture, these cells may be involved in destabilization of the lesion.5 Throughout the progression of atherosclerosis, macrophages produce proinflammatory cytokines, chemokines, growth factors, and matrix-degrading enzymes and are consequently crucial in the chronic inflammatory process in the diseased vessel wall.6,7 Detailed knowledge on the molecular mechanisms involved in the inflammatory and metabolic processes in macrophages is essential to develop novel drug therapies against atherosclerosis. We hypothesized that NR4A nuclear receptors are key regulatory factors involved in modulation of these specific processes in macrophages.

The NR4A nuclear hormone receptors were first described as early response transcription factors expressed on stimulation by growth factors.8–10 This NR4A subfamily comprises 3 members: notably Nur77 (NR4A1, TR3, NGFI-B, NAK-1), Nurr1 (NR4A2, NOT), and NOR-1 (NR4A3, MINOR).11 Like other nuclear receptors, NR4A-factors contain a central DNA-binding domain, comprising 2 zinc-fingers, that bind...
the consensus response element NBRE (AAAGGTC) as monomers and the palindromic NurRE element (TGATATTX6AAAGTCCA) as homo/heterodimers in promoters of specific target genes. Furthermore, nuclear receptors consist of an N-terminal domain mediating transactivation and a C-terminal ligand-binding domain. Specific ligands for the NR4A family of transcription factors have not been identified, classifying them as orphan nuclear receptors. At the C-terminal domain, both Nur77 and Nurr1 can heterodimerize with RXRs and mediate retinoid responses. The NR4A family members have been shown to be functionally involved in T cell and cancer cell apoptosis and in dopaminergic differentiation of neurons.

In searching for genes involved in SMC activation in atherogenesis, we revealed the induction of Nur77 and NOR-1 expression in vitro–activated SMCs. Furthermore, we have shown expression of all 3 NR4A-factors in atherosclerotic lesions and in cultured human SMCs and endothelial cells (ECs). We demonstrated that Nur77 overexpression in vitro inhibits proliferation of both SMCs and ECs. In vivo overexpression of Nur77 under the control of an arterial SMC-specific promoter in transgenic mice protects against SMC-rich lesion formation. Other nuclear receptors, notably PPARs and LXRs, play an important role in both SMC and macrophage function relevant to atherosclerosis and restenosis. However, the function of NR4A-factors in human macrophages is unknown.

In the current study, we show, for the first time to the best of our knowledge, expression of all 3 NR4A family members Nur77, Nurr1, and NOR-1 in human atherosclerotic lesion macrophages, and we demonstrate that these factors reduce the uptake of oxidized low-density lipoprotein (ox-LDL) as well as the inflammatory response in human macrophages.

Materials and Methods

Details of the Materials and Methods are provided in the online data supplement available at http://atvb.ahajournals.org.

Immunohistochemistry and Double In Situ Immunohistochemistry

Macrophages were detected by Ham56 (DAKO) and SMCs by the antibody directed against SMα-actin, 1A4 (DAKO). Anti-Nur77 (M-210), anti-Nurr1 (M-196, Santa Cruz Biotechnology), and anti-NOR-1 (rabbit polyclonal antibody directed against NOR-1 was purchased from Santa Cruz Biotechnology) were used to detect the NR4A-factors. A combination of radioactive gene-specific in situ hybridization and macrophage-specific immunohistochemistry was applied to detect NR4A macrophage-specific expression.

Lentiviral Vector Construction, Infection, and shRNA Interference

hNur77, hNurr1, hNOR-1, and enhanced green fluorescence protein (EGFP) cDNAs were cloned into the pRRL-C-Ppt-PGK-PreSIN vector. Short hairpin (sh) Nur77 and shNOR-1 were cloned into p156RRL-sinPPT-CMV-GFP-PRE/Whel. shRNA design and sequences are available online in the data supplement. Virus was produced as described. THP-1 and U937 cells were transduced for 24 hours with recombinant lentivirus at a multiplicity of infection (MOI) of 3 and 9, respectively, in the presence of 10 μg/mL DEAE-dextran. After transduction, cells were cultured in suspension for 72 hours, differentiated into macrophages, and cultured as described above. Overexpression of Nur77, Nurr1, NOR-1, and EGFP was verified by flow cytometric analyses (EGFP) and immunofluorescence (Figure II,1, available online). shNur77 and shNOR-1 constructs contained CMV-GFP and transduction efficiency was verified by flow cytometric analysis (GFP). Knockdown was confirmed by RT-PCR (Figure II,2, available online) and immunofluorescence.

RNA and Protein Analysis

Briefly, RNA was extracted, and cDNA was made. Specific primers for Nur77, Nurr1, NOR-1, scavenger receptor-A (SR-A), CD36, CD11b, interleukin (IL)-1β, IL-6, IL-8, macrophage inflammatory protein-1α (MIP-1α) and-β (MIP-1β), monocyte chemoattractant protein-1 (MCP-1), and ribosomal protein P0 were designed. Primer sequences are available online in the data supplement. All RT-PCR data were corrected for housekeeping gene ribosomal protein P0.

Lipid Loading, Quantification, and Microscopy

After lentiviral infection THP-1–derived macrophages were treated with DiI-labeled ox-LDL for time periods indicated, subsequently washed twice with PBS, and lysed in isopropanol. After sonification followed by 10 minutes centrifugation (13 000g) DiI-labeled ox-LDL content was measured by fluorometry. For confocal microscopy, cells were cultured on glass and treated with DiI-labeled ox-LDL.

Statistical Analysis

The unpaired Student t test was used to calculate the statistical significance of the expression ratios versus control. P<0.05 was considered statistically significant.

Results

Nur77, Nurr1, and NOR-1 Are Expressed in Human Atherosclerotic Lesion Macrophages

In previous studies we demonstrated expression of Nur77, Nurr1, and NOR-1 in both SMCs and ECs in atherosclerotic lesions. In this study, we show expression of Nur77, Nurr1, and NOR-1 in atherosclerotic lesion macrophages by combining macrophage-specific immunostaining with gene-specific in situ hybridization. Aorta specimens of 8 different organ donors (3 males and 5 females, age 40 to 69 years) were characterized by immunohistochemistry according to the American Heart Association guidelines (Table and Figure 1A and 1B). The complexity of the lesions analyzed ranged from class II to VI. mRNA expression levels of Nur77, Nurr1, and NOR-1 in lesion macrophages and SMCs were scored, and specific localization of expression in the lesion was indicated. As a typical example of an early lesion, a type II lesion with high mRNA expression levels of all 3 nuclear receptors in macrophages was shown (Figure 1C through 1E). Protein expression of Nur77, Nurr1, and NOR-1 localizes to the nucleus in macrophage-rich areas and is comparable with the mRNA expression pattern (Figure 1F through 1I). Notably, in complex lesions, prominent macrophage-specific NR4A expression is localized especially to shoulder regions and macrophages infiltrated in the media.

Nur77, Nurr1, and NOR-1 Are Expressed in Response to Inflammatory Stimuli and Reduce ox-LDL Lipid Loading

High expression levels of NR4A-factors in atherosclerotic lesion macrophages prompted us to study whether their...
expression is dependent on inflammatory signaling pathways that are active at diseased areas. In addition, the functional activity of these transcription factors was determined in vitro studies.

In line with recently published data, we observed robust and transient mRNA expression of all 3 NR4A-factors in primary macrophages and in monocytic THP-1 cells in response to lipopolysaccharide (LPS). In addition, we show that NR4A-factors are moderately induced by tumor necrosis factor (TNF)/H9251 in primary macrophages and highly induced (50- to 150-fold induction) in THP-1 phorbol 12-myristate 13-acetate–maturated macrophages in response to LPS. Immunofluorescent analysis of NOR-1 expression revealed that this protein localizes predominantly to the nucleus after LPS stimulation (Figure III, available online).

To study the function of Nur77, Nurr1, and NOR-1 in macrophages, we infected THP-1 cells with lentiviruses that encode these factors or control Mock-virus and determined the effect on lipid loading, a hallmark of atherosclerosis. Lentiviral overexpression of NR4A nuclear receptors resulted in 80% to 90% transduction efficiency and nuclear localization of the encoded proteins (Figure II.1, available online). Viability of NR4A overexpressing cells was comparable to control cells (data not shown). In macrophages overexpressing NR4A-factors, DiI-labeled ox-LDL uptake was quantified by fluorometry and was shown to be reduced after 3 to 6 hours, with 30% reduction after 24 hours (Figure 2A).

Confocal microscopy was performed to assess the cellular localization of DiI-labeled ox-LDL in macrophages. After 24 hours, DiI-fluorescence localizes to lipid vacuoles and fluorescence intensity is relatively low in Nur77-overexpressing macrophages as compared with Mock-lentivirus–infected cells (Figure 2B). Because SR-A and CD36 are important

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**Figure 1.** A–E, Macrophage-specific expression of Nur77, Nurr1, and NOR-1 in human atherosclerosis. Serial sections of a human type II–lesion († in Table 1), were analyzed by immunohistochemistry to detect macrophages (A) and SMCs (B). To demonstrate macrophage-specific expression of Nur77, Nurr1, and NOR-1, sections were analyzed simultaneously by macrophage-specific immunohistochemistry and in situ hybridization with gene-specific probes (C–E). mRNA expression (black silver grains) of Nur77 (C), Nurr1 (D), and NOR-1 (E) colocalizes with a number of macrophages (in red). F–I, Protein expression of Nur77, Nurr1, and NOR-1 in human atherosclerosis. Serial sections of a human type II–lesion († in Table 1), were analyzed by immuno- histochemistry to detect macrophages (F), Nur77 (G), Nurr1 (H), or NOR-1 (I). NR4A proteins are expressed predominantly in neointimal cells and localize to nuclei. The sections shown in G–I were not counterstained for nuclei. MΦ indicates macrophages; Neo, neointima; Lu, lumen; M, media. Arrows in C, D, and E point at macrophages expressing the specific mRNAs.
genes involved in modified lipoprotein uptake, mRNA expression levels of these genes were determined by semiquantitative real-time RT-PCR. THP-1 macrophages overexpressing Nur77, Nurr1, or NOR-1 express significantly lower levels of SR-A and CD36 than Mock-virus–infected cells (Figure 2C). In addition, we show that CD11b expression, a general macrophage marker gene, is reduced in both THP-1 and U937 macrophages overexpressing NR4A receptors (Figure 2C and 2D). To unravel the function of endogenous NR4A-factors in foam-cell formation, specific shRNAs against Nur77 and NOR-1 were designed. Knockdown of endogenous Nur77 or NOR-1 resulted in a significant increase in DiI-labeled ox-LDL uptake consistent with a 2-fold increase in SR-A and CD36 mRNA expression as compared with cells transduced with control shRNA (Figure 3A and 3B). CD11b expression was increased 2.3- and 2.7-fold in shNur77 or shNOR-1 expressing THP-1 macrophages, respectively (Figure 3C).

**Lentiviral Overexpression of Nur77, Nurr1, and NOR-1 Reduces Proinflammatory Cytokine and Chemokine Expression**

Next, we assayed NR4A function in cytokine and chemokine synthesis in human THP-1 and U937 macrophages. mRNA levels of proinflammatory cytokines IL-1β and IL-6 and chemokines IL-8, MIP-1α/β, and MCP-1 were determined after stimulation with LPS, TNFα, or vehicle (Figure 4A). As a control for the activity of LPS and TNFα, mRNA levels were assayed in Mock-infected macrophages (Figure 4A). Except for IL-6 expression, which is not detectable in vehicle or TNFα-treated cells, mRNA expression levels of these inflammatory genes are induced 20- to 8000-fold by LPS and 3- to 10-fold by TNFα. mRNA levels of these chemokines and cytokines analyzed are robustly reduced (2- to 10-fold) in THP-1 macrophages overexpressing either Nur77, Nurr1, or NOR-1 as compared with Mock-infected cells after LPS and TNFα stimulation. As an exception, MCP-1 mRNA expression is 2.5-fold induced by TNFα in NOR-1 overexpressing macrophages and is not significantly different in Nurr1 overexpressing cells as compared with Mock-infected cells. In addition to the mRNA results described, we determined protein concentrations of IL-1β, IL-6, and IL-8 (Figure 4B) in the conditioned medium of lentivirus-infected THP-1 macrophages. Conditioned media were collected at 0, 6, and 24 hours after treatment with LPS, and protein concentrations were determined by BD Cytometric Bead Array. Overexpression of Nur77, Nurr1, or NOR-1 results in a significant

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** NR4A overexpression in human macrophages reduces Dil-labeled ox-LDL uptake and expression of SR-A, CD36, and CD11b. Uptake of Dil-labeled ox-LDL for 3, 6, and 24 hours was determined by fluorometry. Lipid loading was significantly lower in THP-1 macrophages overexpressing Nur77, Nurr1, or NOR-1 as compared with Mock (A). After 24 hours of Dil-labeled ox-LDL treatment, THP-1 macrophages were analyzed by confocal microscopy showing reduced Dil-fluorescence intensity in Nur77-overexpressing macrophages, localizing to lipid vacuoles (B). mRNA expression of SR-A, CD36, and CD11b was determined by real-time RT-PCR. THP-1 macrophages overexpressing Nur77, Nurr1, and NOR-1 expressed significantly lower levels of SR-A, CD36, and CD11b (C) (A, C; n=3 ± SD, Student t test; P<0.01). In U937 macrophages Nur77, Nurr1, and NOR-1 overexpression resulted in decreased CD11b mRNA expression as compared with Mock (D; n=2 ± SD, Student t test; P<0.02).

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** shRNA-mediated Nur77 and NOR-1 knockdown results in increased Dil-labeled ox-LDL uptake and enhanced expression of SR-A, CD36, and CD11b. Dil-labeled ox-LDL uptake for 24 hours was determined by fluorometry. Ox-LDL uptake was significantly increased in THP-1 macrophages expressing shNur77 and shNOR-1 as compared with control shRNA (A), consistent with a significant increase in SR-A and CD36 mRNA expression as determined by RT-PCR (B) (A, B; n=2 ± SD, Student t test, P<0.05). In addition, in shNur77 and shNOR-1 expressing THP-1 macrophages, elevated CD11b mRNA expression levels were detected as compared with shRNA control (C; n=2 ± SD, Student t test, P<0.05).
reduction of LPS-induced secretion of IL-1β, IL-6, and IL-8 by THP-1 macrophages.

To provide further evidence for an anti-inflammatory function of NR4A-factors in human macrophages, we analyzed cytokine and chemokine expression in human U937 cells in gain-of-function experiments. After stimulation with LPS, NR4A-factors reduce mRNA expression of IL-8 and MCP-1 substantially as well as IL-6 and IL-8 protein levels in conditioned media of these cells (Figure 4C and 4D).

The inhibiting function of endogenous NR4A-factors in inflammatory responses is substantiated by specific shRNAs against Nur77 or NOR-1. Lentiviral delivery of shNur77 or shNOR-1 results in an increase of IL-1β, IL-8, and MCP-1 mRNA expression after LPS stimulation as compared with control shRNA infected cells (Figure 5A). In addition, Nur77 or NOR-1 knockdown significantly increases IL-1β and IL-8 protein concentrations in the supernatant of these cells (Figure 5B).

**Discussion**

Monocyte and macrophage activation together with foam-cell formation are critical events in atherogenesis and other related vascular pathologies. In this study, we demonstrate expression of the NR4A family of nuclear receptors Nur77, Nurr1, and NOR-1 in human atherosclerotic lesion macrophages, especially in areas of plaque activation/progression.

So far, colocalization with macrophage marker CD68 has only been reported for Nur77.30 Lentiviral overexpression of NR4A-factors in human macrophages reduced uptake of modified lipid particles substantially as well as expression of proinflammatory cytokines and chemokines. Moreover, shRNA-mediated knockdown of Nur77 or NOR-1 resulted in increased lipid loading and augmented inflammatory responses in these cells, indicating that endogenous NR4A-factors are involved in these processes. A potential mechanism for the effects observed is inhibition of macrophage differentiation, which is consistent...
with reduced expression of SR-A, CD36, and CD11b in human macrophages in NR4A gain-of-function experiments and enhanced expression of these marker genes in knockdown experiments.

We demonstrated that Nur77, Nurr1, and NOR-1 are transiently induced in response to the inflammatory stimuli LPS and TNFα in both primary and THP-1–derived macrophages. LPS especially, and, as recently shown, also ox-LDL strongly induce NR4A expression. Both LPS and ox-LDL promote Toll-like receptor-4 signaling, which has been shown to be involved in atherogenesis; consequently, these in vitro applied stimuli are relevant to atherosclerosis. Paradoxically, NR4A-factors are expressed in areas of plaque progression/activation and are induced by inflammatory stimuli but, as shown in this study, inhibit foam-cell formation and proinflammatory cytokine as well as chemokine production. Similar atheroprotective mechanisms involved in controlling vascular pathologies have been described for other nuclear receptors and are known to be functional during vascular lesion development.

Nur77 and NOR-1 have been implicated in apoptosis of T cells involving the transcriptional activity of these transcription factors. In macrophages, LPS in combination with the pan-caspase inhibitor zVAD was shown to induce apoptosis involving Nur77, however, the exact mechanism of Nur77 action in zVAD-mediated apoptosis has not been elucidated yet. In cancer cells, the apoptotic effect of Nur77 depends on the presence of proapoptotic agents and involves translocation of Nur77 to mitochondria. Here, we demonstrate nuclear localization of NR4A proteins in human atherosclerotic lesion macrophages as well as in LPS-stimulated cultured macrophages, suggesting the protein to be predominantly active in this cellular compartment. Furthermore, lentiviral overexpression of NR4A nuclear receptors in both THP-1 and U937 macrophages did not result in a reduced viability of those cells.

The reduced uptake of modified LDL as revealed in this study correlates with downregulation of scavenger receptors SR-A, CD36, and CD11b in human macrophages in NR4A gain-of-function experiments and enhanced expression of these marker genes in knockdown experiments.

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Nuclear orphan receptors Nur77, Nurr1 and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses

*Human tissue specimens*

Human tissue samples were obtained with informed consent from organ donors, according to protocols approved by the Medical Ethics Committee of the Academic Medical Center, Amsterdam. The specimens were paraffin embedded, sectioned, and mounted on glass slides (Superfrost-Plus, Emergo). Vascular specimens were characterized by immunohistochemistry with antibodies specific for SMCs and macrophages to establish the stage of disease according to the American Heart Association classification.\(^1\)

*Immunohistochemistry and double in-situ immunohistochemistry*

Macrophages were detected by monoclonal antibody Ham56 (DAKO) and SMCs by monoclonal antibody 1A4 (DAKO) directed against smooth muscle \(\alpha\)-actin, in human vascular specimens. Anti-Nur77 (M-210, Santa Cruz Biotechnology), anti-Nurr1 (M-196, Santa Cruz Biotechnology) and anti-NOR-1 (rabbit polyclonal antibody directed against NOR-1 N-terminal domain was generated; for data on specificity, Figure I, online data supplement) were used to detect NR4A nuclear receptors. Briefly, after deparaffinization and endogenous peroxidase quenching, citrate antigen retrieval was performed, followed by blocking and permeabilization with 1% (w/vol) bovine serum albumin, 1% (vol/vol) normal goat serum and 0.5% Triton-X 100 and primary antibody incubation overnight at 4°C. After biotin-labeled goat-anti-rabbit IgG secondary antibody (DAKO) incubation followed by steptavidin-HRP (DAKO), AEC (Sigma) detection was applied. Staining after secondary antibody incubation alone served as a negative control.
Combination of radioactive gene-specific in situ hybridization and macrophage-specific immunohistochemistry was essentially performed as described. For in situ hybridization the following riboprobes were synthesized: Nur77, GenBank No. L13740, bp 1221 to 1905; Nurr1, GenBank No. X75918, bp 119 to 1003 and NOR-1, GenBank No. U12767, bp 1435 to 2172. After hybridization macrophages were detected using immunohistochemistry as described above, followed by emulsion radiography. Matching sense riboprobes were assayed for each gene and were shown to give neither background nor a non-specific signal. The sections were exposed for 4 to 8 weeks. All slides were counterstained with hematoxylin and embedded in glycergel (DAKO).

Cell culture
Primary human macrophages were isolated from buffy-coats of blood donors, obtained from the Dutch central bloodbank Sanquin. After isolation by Ficoll-Paque (Pharmacia Biotech) gradient centrifugation, monocyte-negative selection kit (Dynal) and adhesion-mediated purification, cells were cultured for 48 hours at a density of 0.5-1x10^6 cells/ml before experiments were performed. Human monocytic THP-1 cells (ATCC) and human monocytic U937 cells were cultured in RPMI 1640, 10% (vol/vol) fetal bovine serum and 100 U/ml penicillin/streptomycin (GIBCO-BRL). Cells were plated in 12-wells plates at a density of 0.5x10^6 cells/ml, differentiated into macrophages by PMA (100 ng/ml) for 48 hours. After differentiation, cells were washed twice with PBS and grown in medium for 24 hours. Reagents used were PMA (Sigma), LPS (Sigma), recombinant human TNFα (R&D) and DiI-labeled ox-LDL (Intracel-RP-173).

Lentiviral vector construction and virus preparation
hNur77 cDNA (GenBank D49728, bp 8-1920) was cloned into the XbaI-NdeI sites of the pRRI-cPpt-PGK-PreSIN vector (PGK-Nur77). hNurr1 cDNA (Genbank X75918, bp 73-2310) was placed into the SalI-NsiI sites of the pRRI-cPpt-PGK-PreSIN vector (PGK-Nurr1) and hNOR-1 cDNA (Genbank D78579, bp 513-2872) was ligated into the XbaI site of the pRRI-cPpt-PGK-PreSIN vector (PGK-NOR-1). PGK-EGFP-PreSIN (PGK-EGFP) was constructed by isolating the EGFP cDNA from the expression vector pEGFP-N2 (Clontech) using SalI-XbaI digestion, subsequently ligated into the corresponding
sites of the pRRl-cPPt-PGK-PreSIN vector. All constructs were verified by DNA sequencing. Virus stocks were produced as described. Briefly, 20 µg of PGK transfer vector, 13 µg of pMDLg/pRRE, 7 µg pVSV-g, and 5 µg of pRSV-REV were co-transfected into 180 cm² HEK293T cells using the calcium phosphate co-precipitation method. Conditioned medium was harvested at 48 hours and 72 hours after transfection, filtered through 0.45µm filters and concentrated by ultra centrifugation (20,000 rpm, 2 hours, 4°C). Viral titers were determined essentially as described by Sastry et al. In short, HEK 293 cells were transduced with serially diluted viral concentrate, 48 hours after transduction total genomic DNA was isolated from these cells and the number of vector DNA copies was determined using PCR analysis with pRRl-cPPt-PGK-PreSIN vector as calibration standard (forward primer: 5’-GTGCAGCAGCAGAACAATTTG-3’, reverse primer: 5’-CCCCAGACTGTGAGTTGCAAATTGTG-3’).

**Lentiviral infection**

THP-1 and U937 cells were transduced in the presence of 10 µgr/ml DEAE-dextran with recombinant lentivirus for 24 hours at a Multiplicity of infection of 3 and 9 respectively. Empty (Mock) and EGFP lentivirus were taken along as controls. After transduction cells were cultured in suspension for 72 hours, differentiated into macrophages and cultured as described above. Overexpression of Nur77, Nurr1, NOR-1 and EGFP was checked by immunofluorescence and flow cytometric analyses (EGFP) (Figure II.1, online data supplement). shNur77 and shNOR-1 constructs contained CMV-GFP and transduction efficiency was verified by flow cytometric analysis (GFP) (data not shown). For immunofluorescence, cells were cultured on glass, fixed for 20 min with 4% (w/vol) paraformaldehyde PBS and permeabilized with 0.5% (vol/vol) Triton-X-100. Cells were stained by anti-Nur77 (M-210, Santa Cruz Biotechnology), anti-Nurr1 (M-196, Santa Cruz Biotechnology) and anti-NOR-1 for detection of Nur77, Nurr1 and NOR-1 respectively, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 568-conjugated donkey anti-goat IgG (Molecular Probes). Nuclei were stained with Hoechst.
RNA Interference

Knockdown of Nur77 or NOR-1 was achieved by lentiviral delivery of an expression cassette encoding an siRNA directed against the target sequences. These sequences are unique to Nur77 and NOR-1, as determined by the Whitehead Institute (Cambridge, MA) siRNA selection program. Briefly, shNur77: CAGTCCAGCCATGCTCCTC TCTCTTGAAAGGAGCACTGGCTGGACTG and shNOR-1: GAAGATCAGACATTACTATCTCCTGAATAAGTAATGTCTGATCTTTT (bold sequences are target sequences; underlined sequences represent hairpin) were coupled to the H1-promoter by PCR amplification and subsequently cloned into p156RRL-sinPPT-CMV-GFP-PRE/NheI as described. Constructs were verified by DNA sequencing. shNur77 and shNOR-1 constructs contained CMV-GFP and transduction efficiency was verified by flow cytometric analysis (GFP). Knockdown was confirmed by RT-PCR (Figure I.2, online data supplement) and immunofluorescence.

RNA and protein analysis

Total RNA was extracted using RNA absolutely Miniprep kit (Stratagene). cDNA was made using iScript cDNA Synthesis kit (Biorad) and semi-quantitative real-time RT-PCR was performed using iQ SYBR-Green Super-Mix in the MyiQ RT-PCR system (Biorad). Specific primers for Nur77, Nurr1, NOR-1, scavenger receptor-A (SR-A), CD36, CD11b, macrophage inflammatory protein-1α (MIP-1α) and-1β (MIP-1β), monocyte chemoattractant protein-1 (MCP-1), IL-8, IL-1β, IL-6 and ribosomal protein P0 were designed as follows:

Nur77: Fw: 5'-gttctctggaggtcatccgcaag-3' Rv: 5'-gcagggaccttgagaaggcca-3'
Nurr1: Fw: 5'-tattccagttccagggca-3' Rv: 5'-gctaatcgaaggacaaacag-3'
NOR-1: Fw: 5'-ccaagccttagcctgcctgtc-3' Rv: 5'-agcctgtccctactctggtgg-3'
IL-1β: Fw: 5'-tgccagaaagggaaacagaaag-3' Rv: 5'-tgtagttggagaggttaggg-3'
IL-6: Fw: 5'-gtgtagttggagaggttaggg-3' Rv: 5'-gtgtagttggagaggttaggg-3'
IL8: Fw: 5'-ctgcgccaacacagaaatta-3' Rv: 5'-attgcctctgtttcactcttc-3'
MIP-1β: Fw: 5'-gctgagctgtcctggtgctc-3' Rv: 5'-gctgagctgtcctggtgctc-3'
MIP-1α: Fw: 5'-gctgagctgtcctggtgctc-3' Rv: 5'-gctgagctgtcctggtgctc-3'
MCP-1: Fw: 5'-ctgagctgtcctggtgctc-3' Rv: 5'-ctgagctgtcctggtgctc-3'
SR-A: Fw: 5’-ctcgctcaatgacagctttgcttc-3’ Rv: 5’-tctttccacctccaggagtttg-3’
CD36: Fw: 5’-gagaactgttatggggctat-3’ Rv: 5’-ttcaactggagaggcaaagg-3’
CD11b: Fw: 5’-cagcacacgcagacagacacag-3’ Rv: 5’-gaggttccgaaagcagacaatgg-3’
P0: Fw: 5’-tcgacaatggcagcatctac-3’ Rv: 5’-atccgtctccacagacaaagg-3’

References


Specificity of the polyclonal anti-NOR-1 antibody. Human NOR-1 amino-acids 1-196 were extended with an N-terminal His-tag and overexpressed in E. coli. After NiTA-purification the protein-fragment was used to raise antibodies against human NOR-1 in rabbits. Serum IgG-fraction was purified by protein-A-Sepharose affinity chromatography.

A: Western blotting. Lysates of COS cells (20μg/lane) transfected with control plasmid or plasmids encoding human Nur77, Nurr1 or NOR-1 under control of the SV40 early promoter were separated by SDS-PAGE and transferred to nitrocellulose membrane. Subsequently, the blot was incubated with anti-NOR-1 antibody (1:1000), followed by goat-anti-rabbit-HRP, which was detected by ECL. The anti-NOR-1 antibody reacted specifically with NOR-1 and shows no cross-reactivity with Nur77 or Nurr1.

B-G: Immunofluorescence. THP-1 cells were infected with empty lentivirus (Mock) or lentivirus encoding human Nur77, Nurr1 or NOR-1 and immunofluorescence was performed with the antibodies indicated. Immunofluorescence and nuclear Hoechst staining overlays are shown (see Materials & Methods). B; THP-1 infected with Mock-lentivirus, analyzed with anti-NOR-1. C; THP-1 infected with NOR-1-lentivirus, analyzed with anti-NOR-1. D, E; THP-1 infected with Nur77- or Nurr1-lentivirus analyzed with anti-NOR-1. F; THP-1 infected with Nur77-lentivirus analyzed with anti-Nur77. G; THP-1 infected with Nurr1-lentivirus, incubated with anti-Nurr1. From these data it can be concluded that the polyclonal anti-NOR-1 antibody specifically recognizes human NOR-1.
Transduction efficiency of lentiviral infection of THP-1 cells, nuclear localization of the encoded nuclear receptors and shNur77 and shNOR-1 efficiency. Figure II.1
THP-1 cells infected with control lentivirus Mock (A, B) or EGFP-encoding lentivirus (C, D) were analyzed by flow cytometry (A-D). Lentiviral infection resulted in 80-90% transduction efficiency. In addition, monocytes THP-1 cells were infected with recombinant lentivirus encoding EGFP (E-G), Nur77 (I-K), Nurr1 (M-O), NOR-1 (Q-S), or with Mock-virus (H, L, P, and T) and differentiated to macrophages by PMA-treatment. Cells were analyzed for direct fluorescence (EGFP and Hoechst) or by immunofluorescence. EGFP protein localized throughout the cell, whereas nuclear receptors are predominantly detected in nuclei. IF, (immuno)fluorescence. Figure II.2
THP-1 cells were infected with shNur77 or shNOR-1 lentivirus containing CMV-GFP with a transduction efficiency of >90% (data not shown), which resulted in a >70% reduction of Nur77 or NOR-1 mRNA expression levels as compared to a control shRNA directed against luciferase (n=2, ±SD, Student’s t-test, p<0.05).

Expression of Nur77, Nurr1 and NOR-1 in primary macrophages and THP-1-derived macrophages in response to LPS and TNFα. mRNA expression levels were determined by real-time RT-PCR. Primary macrophages of 2 different donors were treated with LPS (100ng/ml), TNFα (10ng/ml) or vehicle for 2 hours and substantially increased mRNA expression levels of Nur77, Nurr1 and NOR-1 were observed (A). Also in THP-1-derived macrophages mRNA expression levels of Nur77, Nurr1 and NOR-1 were significantly increased in response to LPS (250ng/ml, 2 hours) (B) and TNFα (10ng/ml, 1 hour for Nur77 and Nurr1, 3 hours for NOR-1) (C). Optimal expression is shown in the upper panels (n=3, ±SD, Student’s t-test, p<0.05) and time courses are given in the lower panels (representative experiment (n=2)). Protein expression of NOR-1 was analyzed after 6 hours LPS in THP-1-derived macrophages by immunofluorescence and NOR-1 protein localized to the nucleus (D). All data shown are significant (p≤0.05) as compared to Mock.
Online supplemental data;
Bonta et al.,
Figure 1
Online supplemental data; Bonta et al., Figure II.1

II.2

Relative expression Nur77 or NOR-1

siCon siNur77 siNOR-1

Mock

EGFP

Overlay

IF Hoechst Overlay Mock overlay

EGFP

Nur77 anti-Nur77

Nurr1 anti-Nurr1

NOR-1 anti-NOR-1
Online supplemental data;
Bonta et al.,
Figure III

**Primary Mø**

- **A**
  - donor 1
  - donor 2
  - Nur77
  - Nurr1
  - NOR-1

- **B**
  - LPS
  - Optimal induction
  - Nur77
  - Nurr1
  - NOR-1

- **C**
  - TNF
  - Fold induction

- **D**
  - NOR-1 expression
  - control
  - LPS

**THP-1 Mø**

- **LPS**
  - Fold induction

- **TNF**
  - Fold induction