NR4A1 (Nur77) Deletion Polarizes Macrophages Toward an Inflammatory Phenotype and Increases Atherosclerosis

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Rationale: NR4A1 (Nur77) is a nuclear receptor that is expressed in macrophages and within atherosclerotic lesions, yet its function in atherosclerosis is unknown.

Objective: Nur77 regulates the development of monocytes, particularly patrolling Ly6C<sup>+</sup> monocytes that may be involved in resolution of inflammation. We sought to determine how absence of nuclear receptor family 4 member A1 (NR4A1) in hematopoietic cells affected atherosclerosis development.

Methods and Results: Nur77<sup>−/−</sup> chimeric mice on a Ldr<sup>−/−</sup> background showed a 3-fold increase in atherosclerosis development when fed a Western diet for 20 weeks, despite having a drastic reduction in Ly6C<sup>+</sup> patrolling monocytes. In a second model, mice deficient in both Nur77 and ApoE (ApoE<sup>−/−</sup>Nur77<sup>−/−</sup>) also showed increased atherosclerosis after 11 weeks of Western diet. Atherosclerosis was associated with a significant change in macrophage polarization toward a proinflammatory phenotype, with high expression of tumor necrosis factor-α and nitric oxide and low expression of Arginase-I. Moreover, we found increased expression of toll-like receptor 4 mRNA and protein in Nur77<sup>−/−</sup> macrophages as well as increased phosphorylation of the p65 subunit of NFκB. Inhibition of NFκB activity blocked excess activation of Nur77<sup>−/−</sup> macrophages.

Conclusions: We conclude that the absence of Nur77 in monocytes and macrophages results in enhanced toll-like receptor signaling and polarization of macrophages toward a proinflammatory M1 phenotype. Despite having fewer monocytes, Nur77<sup>−/−</sup> mice developed significant atherosclerosis when fed a Western diet. These studies indicate that Nur77 is a novel target for modulating the inflammatory phenotype of monocytes and macrophages and may be important for regulation of atherosclerosis.

Key Words: monocyte ■ atherosclerosis ■ nuclear receptors ■ macrophage ■ toll-like receptors

Nur77 is an orphan nuclear receptor that, along with Nurrl and NOR-1, constitutes the nuclear receptor family 4 member A1 (NR4A) subfamily of orphan nuclear receptors in the steroid/thyroid receptor family. They are designated as “orphans" because their ligands are currently unknown. There is some question as to whether these nuclear receptors actually bind ligand, as all 3 receptors appear to have a closed ligand-binding pocket. This has led some to postulate that these receptors are solely regulated in a ligand-independent fashion either transcriptionally or posttranslationally, by kinases and phosphatases.

All 3 NR4A family members are expressed within atherosclerotic lesions. In macrophages, all 3 family members have been identified, and appear to be induced rapidly by multiple stimuli, including lipopolysaccharide (LPS), tumor necrosis factor (TNF)α, and oxidized LDL. Induction of NR4A nuclear receptors leads to rapid induction of transcription of a host of anti-inflammatory genes. These findings have led to the hypothesis that these nuclear receptors act to resolve inflammation. Recently, Nurrl has been shown to serve as a repressor of NFκB activation in microglia and astrocytes. NOR-1 deficiency has been shown to inhibit vascular injury and atherosclerosis in mice. However, the contribution of Nur77 to atherosclerosis development has not yet been reported, although de Vries et al<sup>6</sup> reported that Nur77 agonism inhibited vascular injury.
Monocytes serve as a first line of defense in innate and adaptive immune reactions. They are rapidly recruited to sites of injury or inflammation, where, after differentiation, they can present antigen to T lymphocytes and secrete cytokines. As critical as monocytes are to respond and resolve inflammation, they are also critically involved in mediating chronic diseases, such as atherosclerosis and rheumatoid arthritis. Counterparts to human monocyte subsets have been identified in mice. Two primary monocyte subsets in mice have now been well characterized. One subset is termed as “inflammatory” and the other subset is considered as “surveillance” or “resident.” The inflammatory monocytes are Ly6C+CCR2+CX3CR1+CD62L+ and are selectively recruited to inflamed tissues and lymph nodes. The Ly6C+ inflammatory monocyte subset has been shown to egress from the bone marrow within hours of a bacterial infection in mice, and is CCR2-dependent. The second monocyte subset is characterized as Ly6C-CCR2-CX3CR1+CD62L-. The Ly6C- monocytes patrol the endothelium of blood vessels but may not readily migrate in response to inflammation, except during tissue injury. Several groups have demonstrated that both populations of monocytes are recruited to inflammation or injury sites. Both Ly6C+ monocytes and Ly6C- monocytes can participate in the resolution of inflammation and tissue repair.

Nurrl and NOR-1 have been shown to participate in homeostasis of myeloid progenitors. We recently reported that Nur77 drives expression of the Ly6C- monocyte subset through regulation of differentiation from a myeloid dendritic precursor. Mice deficient in Nur77 showed almost complete loss of Ly6C- monocytes in bone, blood marrow, and spleen. There has been a flurry of reports showing polarization of macrophages in response to tissue injury or infection. In many cases, there is probably a continuum of macrophage phenotypes; however, 2 primary phenotypes have emerged and are delineated as M1 or M2. M1 or classically activated macrophages typically are considered proinflammatory and produce interleukin (IL)-12, TNFα, and nitric oxide. In contrast, M2 “alternatively activated” macrophages express distinct markers such as resistin-like molecule α (RELmα), CD301, CD206, Arginase-I, YM-1, andFizz and may secrete IL-10 or VEGF, among others. Although Ly6C+ and Ly6C- monocytes can differentiate into either M1 or M2-like macrophages, several studies show that Ly6C+ monocytes tend to polarize toward a M1 phenotype in several models, whereas Ly6C- monocytes tend to differentiate into M2-like macrophages. These Ly6C- monocyte-derived macrophages may be involved in resolution of inflammation and wound repair. Interestingly, a recent study indicates that in addition to recently recruited monocytes, macrophages that proliferate locally within tissues can also exhibit a M2 phenotype and control the inflammatory response.

In the current study, we examined the impact of loss of Ly6C+ monocytes in Nur77-/- mice on atherosclerosis development. We report that loss of Nur77 in hematopoietic cells enhances atherosclerosis development, most likely due to differential and enhanced macrophage polarization toward an M1 proinflammatory phenotype.

**Methods**

Detailed, expanded methods can be found online at http://circres.ahajournals.org. C57BL/6J wild-type (WT) mice (006664), ApoE-/- (002052), Ldlr-/- (002207), and Nur77-/- mice on a congenic C57BL/6J background (006187) were from The Jackson Laboratory. Nur77-/- mice were subsequently backcrossed onto the ApoE-/- background. Nur77-/- and WT mice were used as donors for bone marrow transplants into Ldlr-/- recipients as described in the Online Supplement. Atherosclerosis was quantified in the aorta using en face analysis, aortic root analysis, and oil red O staining as previously.

**Results**

**Nur77 Deficiency in Bone Marrow–Derived Cells Increases Atherosclerosis**

We examined atherosclerosis in ApoE-/-/Nur77-/- mice fed a Western diet for 11 weeks. ApoE-/- mice that were deficient in Nur77 showed a significant 4-fold increase in atherosclerosis development by en face staining of the aorta (Figure 1A and 1B). We also observed a 2-fold induction in atherosclerotic lesion area in aortic roots of the ApoE-/-/Nur77-/- mice (Figure 1C). Histological analysis of atherosclerotic lesions showed increased macrophage and lipid content in plaques from ApoE-/-/Nur77-/- mice compared with controls (Figure 1D and 1E). Total cholesterol levels were not different between the 2 groups. There was a trend toward higher plasma triglyceride levels in ApoE-/-/Nur77-/- mice, but this did not reach statistical significance (Online Figure I).

We previously reported that Nur77-/- mice lack Ly6C- monocytes in vivo. We confirmed loss of the Ly6C- monocyte subset in these double knockout mice on the ApoE-/- background. As expected, we found almost complete loss of Ly6C- monocytes in blood and spleens of ApoE-/-/Nur77-/- mice fed a Western diet (Figure 2). In Nur77-deficient mice fed a Western diet, we observed a slight increase in Ly6C- monocytes compared with WT; thus, due to the increase of this subset, overall total monocyte levels were not altered between the 2 groups after Western diet feeding (Figure 2). The T cell compartment was completely normal in Nur77-/- mice (Online Figure II) as has been described. In addition, no differ-
ences in numbers of B cells, granulocytes, or other immune cells were detected in blood from Nur77/H11002/H11002/H11002 mice compared with controls after 11 weeks of Western diet feeding (Online Figure II).

These findings led us to ask whether loss of Nur77 in bone marrow monocyte-derived cells contributes to the increased atherosclerosis observed in these mice. To address this question, we performed bone marrow transplant studies in which WT or Nur77-deficient (Nur77/H11002/H11002/H11002) marrow was transplanted into atherosclerosis-susceptible Ldlr/H11002/H11002/H11002 mice. Mice were allowed to reconstitute the bone marrow compartment for 6 weeks and then were placed on a Western diet for 20 weeks. We observed a 3-fold increase in atherosclerosis development in mice that received Nur77/H11002/H11002/H11002 bone marrow (Figure 3). We also observed a 3-fold increase in the amount of macrophages per aorta in mice that received Nur77/H11002/H11002/H11002 bone marrow (Figure 3C). Thus, absence of Nur77 in bone marrow-derived cells contributes to atherogenesis and increased inflammatory macrophage content, despite the fact that these mice possess significantly fewer Ly6C− monocytes.

Monocytes and Macrophages From Nur77−/− Mice Display a Proinflammatory Phenotype and Show Increased Lipid Accumulation

Based on our previous findings that Nur77 regulates the monocyte compartment in vivo, and based on the strong association of monocytes and macrophages with atherosclerosis, we focused the remaining aspects of our study on understanding how Nur77 regulates monocyte and macrophage function. First, we examined monocyte recruitment to tissues in vivo using a thioglycolate assay in the peritoneal cavity. We did not observe a difference in total populations of resident or thioglycolate-elicited peritoneal macrophages in Nur77/H11002/H11002/H11002 compared with control mice, implying that there is not a major change in macrophage recruitment in the absence of Nur77 (Online Figure III). Second, we examined the inflammatory responsiveness of Ly6C+ monocytes from WT and Nur77−/− mice. Splenic CD115+/CD11b+ cells were gated on Ly6C expression and sorted as described previously.25 Monocytes were used untreated or were stimulated with LPS overnight. Expression of TNFα was quantified in the cells using FACS. Using TNFα protein expression as an

Figure 1. Increased atherosclerotic plaque area in ApoE−/−Nur77−/− mice. A, Quantification of plaque area as percentage of aortic surface in ApoE−/−Nur77−/− mice and ApoE−/− control mice after 11 weeks of Western diet feeding. P<0.01 (Mann-Whitney test). B, Representative oil red O staining (red) in aortic arches of ApoE−/−Nur77−/− mice and ApoE−/− control mice after 11 weeks of Western diet feeding. C, Quantification of aortic root lesion area in ApoE−/−Nur77−/− mice and ApoE−/− control mice after 11 weeks of Western diet feeding. P<0.02 (Student t test). D, Oil red O (red, top) and CD68+ macrophage (green, bottom) staining of aortic root sections from ApoE−/− (left) and ApoE−/−Nur77−/− (right) mice on 11 weeks of Western diet feeding under low magnification (<×10, left panel of each group) and high magnification (×40, right panel of each group). E, Quantification of oil red O (left) and macrophage (CD68, right) staining expressed as percentage of total plaque area. *P<0.05 (unpaired Student t test). Data are representative of 2 independent experiments (n=12 per group for A and B, n=7 per group for C, and n=10 per group for D and E; values are mean±SEM).
indicator of inflammation, we observed that Ly6C⁺ monocytes from Nur77-deficient mice showed an exacerbated response to LPS (Figure 4A). To examine if this exaggerated LPS response in Ly6C⁺ monocytes could be possibly related to an absence of Nur77 in this subset, we examined the ability of WT Ly6C⁺ cells to upregulate Nr4A1 family members in response to inflammatory stimuli and on adhesion-induced differentiation. As macrophages have previously been shown to rapidly upregulate Nr4A1 family members in response to LPS, we confirmed this phenomenon in peritoneal macrophages (Online Figure IV) and determined that circulating Ly6C⁺ monocytes likewise upregulate Nur77 and NOR1, but not Nurr1, in response to LPS stimulation (Figure 4B). Thus, Nur77 is induced in Ly6C⁺ monocytes on inflammatory stimulation, which suggests that Nur77 has a function in the stimulated monocyte. As the inflammatory response is exacerbated in the Ly6C⁺ monocyte lacking Nur77, this suggests that at least one function of Nur77 in the activated Ly6C⁺ monocyte may be to resolve or control the inflammatory response of this cell during inflammation. In addition, monocytes adhere and will differentiate into macrophages when incubated on plastic. Using a new reporter transgenic mouse line transgenic for Nur77 (Figure 2), we evaluated the influence of Nur77 deficiency in atherosclerosis development. ApoE⁻/⁻ Nur77⁻/⁻ mice fed a Western diet showed a significant reduction in the Ly6C⁺ monocyte population in the blood and spleen (Figure 2). This finding supports a role for Nur77 in the regulation of monocyte number in vivo. To further examine the role of Nur77 in monocyte/macrophage biology, we transplanted bone marrow from Nur77⁻/⁻ or WT mice into Ldlr⁻/⁻ recipient mice and quantified atherosclerotic plaque area and macrophage content in the aortic arch after Western diet feeding. Nur77⁻/⁻ bone marrow recipients showed a significant increase in atherosclerotic plaque area and macrophage content compared to WT bone marrow recipients (Figure 3). These data suggest that Nur77 plays a role in the regulation of monocyte/macrophage biology and potentially in the development of atherosclerosis.

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ApoE showed a slight but significant increase in expression of Arginase-I (ArgI) in ApoE/H11002 (WT) and ApoE/Nur77−/− (Nur77−/−) mice were cultured for 18 hours in the absence (UN) or presence of 50 ng/mL LPS. Cells were stained intracellularly for TNFα and analyzed by flow cytometry. Data are expressed as fold change in TNF mean fluorescent intensity over Ly6C WT monocytes set as 1. Data are representative of 2 independent experiments (n=3 per group; values are mean±SEM). *P<0.001 (unpaired Student t test). B, Induction of Nr4A1 family members Nur77, NOR1, and Nur1 in C57BL/6J control Ly6C blood monocytes in response to LPS (100 ng/mL) stimulation for 1.5 hours (n=4 per group; values are mean±SEM). *P<0.001 (unpaired Student t test). Quantification of data expressed as percent change in mRNA of LPS-treated over untreated expression for each gene set as 100%. C, Isolated Nur77−/− GFP+CD11b+ Ly6C+ inflammatory monocytes with almost no initial GFP expression (Untreated) achieved increased GFP expression after 2 hours incubation on plastic. Inflammatory Nur77−/− GFP+CD11b+ Ly6C+ monocytes were sorted from blood using a FACS Aria II cell sorter, and isolated cells were incubated for 2 hours at 37°C, 5% CO2 on a plastic surface. Negative control is the same monocyte population from a mouse. Data are representative of 3 independent experiments.

We next measured the ability of Ly6C+ monocytes to accumulate lipid. Ly6C+ monocytes isolated from Ldlr−/− recipients that had received Nur77−/− bone marrow showed increased neutral lipid accumulation as measured by Nile red staining (Figure 5A). Ly6C+ monocytes lacking Nur77 showed a slight but significant increase in expression of SR-A but not CD36 (Figure 5B). Culturing the Ly6C+ monocytes overnight with Dil–oxidized low-density lipoprotein (OxLDL) resulted in increased uptake of OxLDL in the Nur77−/− monocytes compared with WT (Figure 5C). Thus, Ly6C+ monocytes lacking Nur77 have enhanced cytokine production and enhanced uptake of OxLDL, indicating a proinflammatory phenotype.

We then examined the phenotypes of macrophages in these mice. We isolated resident macrophages from peritoneal lavage of 11-week Western diet–fed ApoE−/− and ApoE−/− Nur77−/− mice (gating strategy, Online Figure V) and found enhanced expression of IL-12 and reduced expression of Arginase-I (ArgI) in ApoE−/−/Nur77−/− macrophages compared with ApoE−/− (WT) resident macrophages (Figure 6A). Stimulation of these macrophages from 11-week Western diet–fed mice for 2 hours with Kdo Lipid A (KLA), the active component of LPS, caused further induction of IL-12, TNF, and iNOS in WT cells (Figure 6A). The IL-12 and iNOS expression in the stimulated macrophages was tripled in the absence of Nur77. ArgI expression remained lower in the ApoE−/− Nur77−/− macrophages, even when treated with KLA. MHCII expression was significantly higher on peritoneal macrophages isolated from ApoE−/− Nur77−/− mice compared with ApoE−/− (Figure 6B), supporting the notion of increased macrophage activation in the absence of Nur77. We also observed increased Dil–OxLDL uptake in bone marrow–derived macrophages cultured in GM-CSF from Nur77−/− mice (Figure 6C). Finally, to confirm that macrophages in aorta of ApoE−/− Nur77−/− mice showed a similar proinflammatory phenotype, F4/80+ macrophages from aorta were analyzed for CD36, SR-A and TNFα expression. We observed a significant 2-fold induction of TNFα, a 2.5-fold induction of SR-A, and a 5-fold induction of CD36 in ApoE−/− Nur77−/− macrophages obtained from aorta (Figure 6D), supporting the presence of proinflammatory macrophages and increased lipid uptake in the aorta.

A recent study by Herzenberg et al37 reported the presence of small versus large macrophages in the peritoneal cavity. Although Jenkins et al38 have recently reported that macrophages can proliferate in tissue rather than necessarily being recruited from blood or spleen, many macrophages are indeed recruited to tissues in response to inflammatory stimuli. Small macrophages are thought to be derived from recruited Ly6C+ monocytes, whereas large macrophages tend to be the resident macrophages. In the peritoneal cavities of ApoE−/− Nur77−/− mice, we found fewer large macrophages and more small macrophages (Online Figure VI).

Abnormal Toll-Like Receptor Expression and NFκB-Mediated Signaling in Nur77−/− Macrophages

Interestingly, we found that expression of several toll-like receptors was increased in ApoE−/− Nur77−/− macrophages fed chow and Western diets, particularly toll-like receptor (TLR)4 and TLR9 (Figure 7A). Concomitant with increased TLR4 mRNA expression, we found increased TLR4 surface protein expression in macrophages from ApoE−/− mice.
lacking Nur77 by FACS (Figure 7B). Nur77-deficient macrophages also showed increased expression of IL-12, and iNOS mRNA after 2 hours of KLA incubation in vitro (Figure 7C). There was a small increase in TNFα expression although it was not significant. These data point to polarization of macrophages toward a M1 phenotype in the absence of Nur77. Moreover, Nur77-deficient macrophages exhibited a blunted “M2-like” response, with little induction of Arg1 expression in response to KLA treatment (Figure 7C). Similarly, Nur77-deficient macrophages showed enhanced responsiveness to TLR agonists, including the TLR4 agonist KLA, the TLR2 agonist Pam3CSK4, and the TLR7 agonist R-848 (Figure 7D). Treatment with these TLR agonists uniformly increased TNFα, IL-12, and nitric oxide production (Figure 7D).

We also discovered that, similar to what we previously reported for monocytes, Nur77-deficient macrophages had increased phosphorylation of the p65 subunit of NFκB, suggesting enhanced activation of NFκB (Figure 7E). Phosphorylation of p65 helps stabilize NFκB in the nucleus for gene transcription.39,40 To determine if the enhanced inflammatory signaling in Nur77-deficient macrophages is NFκB-dependent, we conducted similar peritoneal macrophage activation experiments in the presence of the NFκB inhibitor BAY11–7082 (BAY), which selectively and irreversibly inhibits NF-κB activation by blocking phosphorylation and degradation of IκB-α. The increased inflammatory activity observed in the Nur77-deficient macrophages was ablated by BAY-mediated inhibition of NF-κB (Figure 7F and 7G). Thus, Nur77-deficient macrophages show enhanced NFκB-dependent inflammatory activation.

**Human CD14dimCD16+ Monocytes Express Nur77**

Three distinct subsets of human monocytes have been identified, based on their surface markers: CD14+CD16−, CD14+CD16+, and CD14dimCD16+ (Figure 8A). Murine Ly6C+ monocytes are analogous to CD14+ monocytes in humans, which exhibit a strong inflammatory response to LPS, and Ly6C− mouse monocytes are most analogous to CD14dimCD16+ human monocytes, which patrol blood vessels.11 The function of the newly identified CD14+CD16+ subset is unclear.11,40 We quantified Nur77 mRNA expression in these 3 human monocyte subsets using real-time PCR and found a pattern of expression...
similar to what we observe in the mouse. CCL3 is highly expressed in CD14$^{hi}$ monocytes and is shown here as a control for separation of the subsets. Expression of NR4A1 is quite high in CD14$^{dim}$CD16$^{+}$ human monocytes, the counterpart to patrolling Ly6C$^{-}$ monocytes in mice (Figure 8B). Nur77 was expressed in CD14$^{+}$CD16$^{+}$ human monocytes, but at much lower levels. In contrast, Nur77 was barely detectable in the CD14$^{+}$ subset (analogous to Ly6C$^{+}$ monocytes in mice). Thus, Nur77 probably plays a functional role in the CD14$^{dim}$CD16$^{+}$ monocyte subset in humans under basal conditions, similar to mice.

**Discussion**

The NR4A family of nuclear receptors plays an important role in inflammation and vascular injury. Although studies have shown that NOR-1 is important in atherogenesis, the role of Nur77 in atherogenesis has remained unclear. We recently reported a role for Nur77 in regulating monocyte development, and others have reported a role for NR4A family members in regulating inflammatory responses in macrophages and microglia. In the current study, we show that mice deficient in Nur77 display greater atherosclerosis development than do control mice when fed a Western diet. Our data indicate that this is associated with (1) a lack of Ly6C$^{-}$ patrolling monocytes and (2) enhanced polarization of Ly6C$^{+}$ monocytes and derived macrophages toward a proinflammatory M1-like phenotype, which may be responsible for exacerbated lesion development. How enhanced M1 polarization is related to loss of Ly6C$^{-}$ monocytes remains to be elucidated.

Based on our prior work that showed the almost complete loss of Ly6C$^{-}$ monocytes in Nur77$^{-/-}$ bone marrow, blood, and spleen, one hypothesis is that the increased macrophage activation and phenotypic proinflammatory M1 polarization of macrophages in Nur77$^{-/-}$ mice is due to loss of this protective Ly6C$^{-}$ monocyte subset. Lack of this subset may impair the resolution of inflammation and would lead to enhanced proinflammatory signaling in macrophages, thus diminishing an M2-like inflammatory response by macrophages. A second possibility for the observed inflammatory response...
Figure 7. Increased TLR expression and NFκB-mediated inflammatory cytokine production by Nur77−/− macrophages. A, Expression of TLR2, 4, 7, and 9 mRNA levels in peritoneal macrophages from ApoE−/− Nur77−/− (Nur77−/−) mice and ApoE−/− control (WT) mice on chow (left), after 11 weeks of Western diet feeding (center), or after Western diet feeding with incubation for an additional 2 hours with 30 μg/mL KLA (right). Quantification of data expressed as percent change in Nur77−/− mRNA over WT expression for each gene. B, Representative flow plot (left) and quantification (right) of TLR4-MD2 expression on peritoneal macrophages from ApoE−/− Nur77−/− (Nur77−/−) mice and ApoE−/− control (WT) mice measured by flow cytometry. Quantification of data expressed as fold change in TLR4-MD2 mean fluorescent intensity over WT macrophages set as 1; *P<0.01. C, IL-12, TNFα, iNOS, and Arginase (Arg1) mRNA expression in peritoneal macrophages from Nur77−/− mice and C57BL/6J wild-type control (WT) mice on chow diet, unstimulated (UN), or stimulated for 2 or 24 hours with 30 μg/mL KLA (right) measured by quantitative real-time PCR. Quantification of data expressed as percent change in mRNA over untreated WT expression for each gene. D, TNFα, IL-12, and nitric oxide production in production by peritoneal macrophages from Nur77−/− mice and C57BL/6J wild-type control (WT) mice on chow diet, unstimulated (UN) or stimulated for 18 hours with either 30 μg/mL KLA, 5 μg/mL R848, or 30 ng/mL PAM2. TNFα and IL-12 proteins were measured by ELISA, and nitric oxide was measured by the Griess assay. E, Intracellular staining of p65 activation in peritoneal macrophages from Nur77−/− mice and C57BL/6J wild-type control (WT) mice on chow diet unstimulated (UN) or stimulated for 2 hours with either 30 μg/mL KLA by flow cytometry using a p65 phosphospecific antibody. Data are representative of 2 independent experiments. F, IL-12, iNOS, and TNFα mRNA expression in peritoneal macrophages from Nur77−/− mice and wild-type control (WT) mice unstimulated (UN), pretreated for 1 hour with the NFκB inhibitor Bay11–7082 (Bay), stimulated with 100 ng/mL LPS (LPS) for 2 hours, or stimulated with LPS after Bay11–7082 pretreatment (LPS+Bay), measured by quantitative real-time PCR. Quantification of RNA data expressed as percent change in Nur77−/− mRNA over WT expression for each gene. G, Quantification of TNFα protein levels secreted in media by peritoneal macrophages treated under same conditions as in A. *P<0.01 (unpaired Student t test) (n=6 per group; values are mean±SEM) (n=6, A through C, F, and G; D and E, n=4 per grouping; values are mean±SEM).
The macrophage phenotype is that Ly6C<sup>+</sup> monocytes from Nur77<sup>−/−</sup> mice may give rise to a more proinflammatory activated M1-like macrophage phenotype, suggesting that Nur77 plays a cell-autonomous role in regulating the inflammatory responsiveness of the Ly6C<sup>+</sup> monocyte subset. This scenario would be independent of any regulation or suppression by Ly6C<sup>+</sup> monocytes. In support of this concept is the fact that Nur77, although highly expressed in Ly6C<sup>+</sup> monocytes, is also present in Ly6C<sup>+</sup> monocytes, particularly on their activation (Figure 4B). Therefore, Nur77 probably becomes important in activated monocytes and macrophages in a cell-autonomous manner to keep the inflammatory process in check. In the absence of a Nur77-mediated negative feedback mechanism, these macrophages appear to be somewhat constitutively activated and hypersensitive to inflammatory stimuli. However, we cannot rule out an important role solely for the Ly6C<sup>−</sup> monocyte subset in resolving the inflammatory response in vivo, particularly in that the known function of this subset is to patrol the vasculature in search of pathogens. This subset could act to directly dampen an inflammatory response that is mediated by the Ly6C<sup>+</sup> subset in vivo. Future studies will hopefully allow us to discern the roles of each of these monocyte subsets on atherogenesis as well as to define how Nur77 influences the function of each subset.

In the current study, we observed increased inflammatory activities mediated via activation of the NFκB and TLR signaling pathways in Nur77<sup>−/−</sup> macrophages, which are blocked by NFκB inhibitors (Figure 7). We do not find any evidence of Nur77 activation of NFκB signaling by induction of IKK expression as has been reported by Pei et al. The studies from Pei et al were Nur77 overexpression studies performed in macrophage cell lines and not in primary cells or in vivo, which may partially explain the differing results. As of yet, we do not know the exact mechanisms for how Nr4a1 functions in these cells to regulate NFκB. We believe the increased inflammatory activity is at least partially due to the absence of Nur77’s ability to produce the NFκB inhibitory protein, IκB-α, as we report in monocytes and others have reported in cell lines. Nur77 has been demonstrated to positively regulate expression of IκB-α via a defined and functional Nur77 response element in the IκB-α promoter. Glass et al have also previously shown that another NR4A family member, Nurr1, inhibits NFκB through the recruitment of corepressor CoREST complexes, in microglia. This Nurr1-CoREST negative feedback loop results in NFκB turnover and loss of activated gene expression. Whether Nur77 interacts with CoREST in macrophages is currently unknown. Additionally, the ability of Nur77 to heterodimerize with other nuclear receptors such as RXR allows for varying cellular responses and probably will make the underlying mechanism of Nur77 regulation of NFκB and other proinflammatory transcription factors more difficult to discern.

As a transcription factor, Nur77 functions to regulate many cellular processes, including cell growth, activation, proliferation, and apoptosis. TLR-mediated NFκB signaling is one of several pathways that may be modulated by this nuclear receptor. Increased NFκB activity in the absence of the Nur77 probably induces expression of a number of proinflammatory cytokines due to the loss of Nur77 transrepression. Indeed, studies have suggested that...
Nur77 negatively regulates several cytokines that may be important in inflammation.\textsuperscript{44–46} Previous studies have demonstrated that Nur77 expression inhibits scavenger receptor-A and CD36 expression to reduce lipid loading in cultured macrophages, implying a role in regulating lipid uptake.\textsuperscript{46} We confirm this important role of Nur77 in regulating lipid uptake by demonstrating increased scavenger receptor expression and lipid uptake in macrophages in the absence of Nur77 in vivo. We also find a role for Nur77 in regulating scavenger receptor-A expression and lipid uptake in monocytes. Interestingly, we did not observe variation of CD36 expression in monocytes as we did in macrophages. One possible explanation of this result is that there may be differential regulation of these scavenger receptors in monocytes versus macrophages. In fact, it has been demonstrated that CD36 is strongly upregulated with monocyte to macrophage differentiation and M-CSF stimulation.\textsuperscript{47} In addition, Nur77 can function to directly induce expression of anti-inflammatory genes. One example of this is that Nur77 has been shown to induce ABCA1, a protein involved both in cholesterol efflux and macrophage inflammation.\textsuperscript{41,48,49} ArgI is a well-known marker of macrophage polarization whose expression is increased in anti-inflammatory M2 macrophages. As such, we find reduced expression of ArgI in Nur77\textsuperscript{+/−} macrophages (Figure 7). Analysis of the murine ArgI promoter using Jaspar and MacVector software packages revealed the presence of 8 putative Nur77 NBRE elements and one NBRE:RXR element located within a 3-kb fragment upstream of the transcriptional start site of the murine ArgI gene (data not shown). Thus, Nur77 may directly regulate ArgI transcription, either alone or through RXR dimerization, which would directly impact the polarization of macrophages.

We focused on macrophages in this study because of our recent finding that Nur77 regulates monocyte development. In the current study, we find increased macrophage inflammation in atherosclerotic plaques of Nur77\textsuperscript{−−/−} mice, confirming the importance of Nur77 in regulating inflammatory events in myeloid cells. However, Nur77 also influences T cell receptor (TCR) affinity,\textsuperscript{35} thereby possibly regulating T cell activation or even thymocyte selection. Several groups have implicated Nur77 in thymocyte selection,\textsuperscript{30,51} although the Nur77\textsuperscript{+/−} mouse shows normal T cell development.\textsuperscript{34} We observed no obvious differences in numbers of circulating T cells or other immune cells in Nur77\textsuperscript{−−/−} mice fed a Western diet (Online Figure II), implying that the increase in atherosclerosis is probably myeloid cell-specific. Furthermore, under normal conditions Nur77 is most highly expressed in Ly6C\textsuperscript{−} monocytes, with little expression in other immune cell types.\textsuperscript{25,35} Nur77 may be upregulated in lymphocytes including T cells, NKT, and T regs with antigen stimulation but not with inflammatory stimuli, implying that there might not be a major role of Nur77 in T cell–mediated inflammation.\textsuperscript{35} Although our results clearly point to a critical role for Nur77 in monocytes and macrophages, we cannot definitively rule out a role for Nur77 in T cells or other immune cells on atherosclerosis development. The use of conditional knockout mice will be important to delineate the roles of lymphocyte versus myeloid-selective Nur77 functions in atherogenesis.

The fact that we observed high expression of Nur77 in CD14\textsuperscript{dim} human monocytes, the equivalent to the Ly6C\textsuperscript{−} patrolling monocyte subset in mice, suggests perhaps a unique function for Nur77 in this human monocyte subset. Polymorphisms in NR4A1 that change Nur77 expression could be important for both acute and chronic inflammatory responses in human disease. Several SNPs have been reported for the human NR4A1 gene and several of these are located within the known promoter region of the gene.\textsuperscript{52} Future studies of these polymorphisms in population studies will be important for determining whether therapies targeting Nur77 expression could be effective for inflammatory diseases.

In summary, we have identified that absence of Nur77 in hematopoietic cells amplifies atherosclerosis development in mice. Our data suggest that Nur77 functions to regulate macrophage phenotypes in vivo and that absence of Nur77 in macrophages skews the profile of macrophages toward a proinflammatory, proatherogenic phenotype.

Acknowledgments
We acknowledge Amy Blatchley and Deborah Yoakum for mouse colony management and Jennifer Pattison and Dr Joseph L. Witztum for assistance with plasma lipoprotein analysis.

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Disclosures
None.

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**Novelty and Significance**

**What Is Known?**
- Monocyte-derived macrophages and foam cells are the primary cell
types that constitute atherosclerotic plaques.
- Multiple studies have shown that the inflammatory phenotype of
macrophages or foam cell formation increases atherosclerotic
plaque size.
- The orphan nuclear receptor Nur77 has the ability to suppress
inflammatory activity and is involved in regulation of monocyte
and macrophage production.

**What New Information Does This Article Contribute?**
- Mice transplanted with Nur77-deficient bone marrow–derived cells
show increased aortic lipid accumulation associated with in-
creased macrophage content in plaques.
- Nur77-deficient macrophages have increased inflammatory activity
and exhibit increased lipid uptake.
- Nur77-deficient macrophage inflammatory activity is
NFκB-dependent.

Monocytes are the primary inflammatory cell type that infiltrates
early atherosclerotic plaques, and their recruitment into plaques
drives disease progression. We have found that the orphan nuclear receptor Nur77 functions as a master regulator of the
differentiation and survival of a unique monocyte subset (Ly6C–
in mice), which patrols the vasculature and participates in the
resolution of inflammation. Here, we report that mice deficient in
Nur77 show increased inflammatory activity and atherosclerotic
development. Nur77-deficient macrophages exhibited a proin-
flammatory M1-like phenotype, in which inhibiting NFκB could
block their activation. These findings suggest that hematopoietic
expression of Nur77 can suppress polarization of macrophages
to a proinflammatory phenotype and that absence of the
patrolling monocytes exacerbates inflammation, most likely due
to an inability to resolve the inflammatory response. Thus, our
study identifies Nur77 as a novel target for modulating the
inflammatory phenotype of monocytes and macrophages in
cardiovascular diseases.
NR4A1 (Nur77) Deletion Polarizes Macrophages Toward an Inflammatory Phenotype and Increases Atherosclerosis

Richard N. Hanna, Iftach Shaked, Harper G. Hubbeling, Jennifer A. Punt, Runpei Wu, Erica Herrley, Claudia Zaugg, Hong Pei, Frederic Geissmann, Klaus Ley and Catherine C. Hedrick

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Methods.

Mice. C57BL/6J wild-type mice (000664), ApoE<sup>−/−</sup> (002052), Ldlr<sup>−/−</sup> (002207), and Nur77<sup>−/−</sup> mice on a congenic C57BL/6J background (006187) were from The Jackson Laboratory. Nur77<sup>−/−</sup> mice were subsequently backcrossed onto the ApoE<sup>−/−</sup> background. Nur77<sup>−/−</sup> and wild-type mice were used as donors for bone marrow transplants (BMTs) into Ldlr<sup>−/−</sup> recipients (BMT protocol described below). Mice were maintained on a standard rodent chow diet or fed a Western-type diet (Harlem TD.88137) for eleven weeks (ApoE<sup>−/−</sup> mice) or twenty weeks (Ldlr<sup>−/−</sup> mice). Nur77-GFP mice were generated as described<sup>1</sup>. Mice were housed in microisolator cages in a pathogen-free facility. All experiments followed guidelines of the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee, and approval for use of rodents was obtained from the La Jolla Institute for Allergy and Immunology according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Mice were euthanized by CO2 inhalation.

Bone marrow transplantation studies. Recipient mice (Ldlr<sup>−/−</sup>) were irradiated in two doses of 550 rads each (for a total of 1,100 rads) 4 h apart. Bone marrow cells from both femurs and tibias of donor mice (wild-type or Nur77<sup>−/−</sup>) were collected under sterile conditions. Approximately 5 x 10<sup>7</sup> nucleated bone marrow cells were obtained from each donor mouse. Bones were centrifuged for the collection of marrow, then cells were washed and resuspended in Dulbecco’s PBS for injection. Approximately 5 x 10<sup>6</sup> unfractionated bone marrow cells in 200 µl media were delivered retro-orbitally into each recipient mouse. Recipient mice were housed in a barrier facility under pathogen-free conditions before and after bone marrow transplantation. After bone marrow transplantation, mice were provided autoclaved acidified water with antibiotics (trimethoprim-sulfamethoxazole) and were fed autoclaved food for 4 weeks. Mice were used in studies or underwent Western diet feeding after 6 weeks of bone marrow reconstitution.

Atherosclerosis Quantification. Atherosclerosis was quantified in the aorta using en face analysis, aortic root analysis and Oil Red O staining as described previously<sup>1,2</sup>.

Plasma Cholesterol Measurements. Plasma cholesterol and triglycerides were measured using FPLC as described previously<sup>2,3</sup>.

Histology. Aortic roots were frozen in OCT media and sectioned (10µm) on a cryostat. Sections were dried for 30 min at room temperature, fixed for 20 min in 2% paraformaldehyde, washed 3 times in PBS, then stained in Oil-Red-O solution for 20 min, washed in 60% isopropanol for 10 seconds, washed for 3 min in running deionized water, permeabilized in 0.2% citrate/0.2% Triton X in PBS for 5 min, washed for 3 times in PBS, blocked in 5% goat serum with 0.2% Triton X in PBS for 60 min, and then incubated in anti-CD68-FITC antibody diluted in PBS with 1% BSA and 0.2% Triton X overnight at 4 °C. The next day slides were washed 3 times with PBS, then incubated with anti-FITC antibody (Invitrogen) in PBS with 1% BSA and 0.2% Triton X for 60 min at room temperature, then washed 3 times in PBS, and mounted in Prolong Gold w/DAPI (Invitrogen). Slides were imaged with a FluoView FV10i confocal laser-scanning microscope (Olympus). Percentage of macrophage (CD68<sup>+</sup>) and lipid (Oil-Red-O<sup>−</sup>) area in aortic root plaques was calculated using Adobe Photoshop and ImageJ software. Plaque regions were cut out in Photoshop and then analyzed in ImageJ for percentages of fluorescent CD68 and Oil-Red-O staining area per total plaque area.

Flow cytometry. Spleens were excised and pushed through a 70-µm strainer, peritoneal macrophages were collected by peritoneal lavage, and blood was obtained by cardiac puncture with an EDTA-coated syringe. All samples were collected in Dulbecco’s PBS (Gibco) with 2 mM EDTA and were stored on ice during staining and analysis. Red blood cells were lysed in RBC Lysis Buffer according to the manufacturer’s protocol (BioLegend). Cells (2 x 10<sup>6</sup> to 4 x 10<sup>7</sup>) were resuspended in 100 µl flow staining buffer [1% BSA (wt/vol) plus 0.1% (wt/vol) sodium azide in PBS]. Fc receptors were blocked for 15 min and surface antigens on cells were stained for 30 min at 4 °C (flow cytometry antibody clones used are shown in Online Table 1). LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) was used for analysis of viability, and forward- and side-scatter parameters were used for exclusion of doublets from analysis.
For measurement of intracellular lipids by Nile Red staining, cells were stained for an additional 15 min at RT in PBS with 200 ng/µl nile red (Enzo) and then washed one time in PBS before analysis following established methods.  

For intracellular staining of TNFα and p65 (pS529), cells were fixed and made permeable with the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). Cells were stained for 30 min at 4 °C with directly conjugated fluorescent antibodies (Online Table I). Calculations of percentages were based on live cells as determined by forward and side scatter and viability analysis. Mean fluorescence intensity was quantified, and expression was calculated relative to that of the wild-type control. Monocytes were identified as Lin− (CD19, CD3ε, Ly6G, CD49b, MHCI), CD115+, CD11b+ and then identified as Ly6C+ or Ly6C− as previously described. Peritoneal macrophages were identified as described in Online Figure V, based on the gating strategy of Ghosn et al. Cellular fluorescence was assessed using LSR II, FACSAria II or FACSCalibur instruments (all from BD Biosciences) and data were analyzed with FlowJo software (TreeStar version 9.2).

**Cell sorting.** In most experiments, spleens were enriched for monocytes by negative selection with isolation kits according to the manufacturer’s protocol (CD19, CD4, CD8, Ter119 custom ‘cocktail’ for monocytes, Miltenyi Biotec) before cell sorting. Cell sorting of monocyte populations from the Nur77-GFP reporter mouse was performed on whole splenocytes. Peritoneal cells were collected by lavage and sorted for large and small macrophages using the gating strategy in Supplemental Fig 2. Surface antigens on cells were then stained as described above, followed by cell sorting with a FACSAria cytometer (BD Biosciences). Approximately 5 x 10⁴ to 10 x 10⁶ events were collected for mRNA samples. The purity of monocyte was verified by microscopy of cytospin preparations of sorted cells stained with HEMA 3 (Fisher Scientific).

**Aortic flow cytometry.** Aorta flow sorting was conducted as described. In brief, anesthetized mice were perfused by cardiac puncture with DPBS containing 2 mM of EDTA. Harvested aortas were digested with 125 U/mL collagenase type XI, 60 U/mL hyaluronidase type I-s, 60 U/mL DNAse1, and 450 U/mL collagenase type I (all enzymes, Sigma) in PBS containing 20 mmol/L HEPES at 37 °C for 1 hour. A cell suspension was obtained by mashing the aorta through a 70-µm strainer. Cells were then stained for flow cytometry as described above. Macrophages were identified as live, F4/80+, CD11b+, CD45+, CD19−, CD3ε−, CD11c− cells using a FACSAria cytometer. Sorted cells were immediately washed with DPBS, and frozen at -80 °C in lysis buffer for RNA extraction and PCR analysis as described below.

**Monocyte, spleen and peritoneal macrophage culture.** Sorted Ly6C+ and Ly6C− monocytes were washed once in DPBS and cultured for 18 hours with or without 50 ng/ml LPS (Sigma, L2018) in Iftach’s media (RPMI, HEPES 1%, Anti Anti 1%, 2-Mercaptoethanol 1%, non essential amino acids 1%, Na pyruvate 1%, heat-inactivated FBS 10%) with Golgi-stop (BDbiosciences). Cells were then re-stained for flow cytometry as described above with the same surface antibodies, and then stained intracellularly for TNFα. Sorted monocyte populations from the Nur77-GFP reporter mouse were incubated on plastic tissue culture treated plates from Costar (#3534) for 2 hours before analysis by flow cytometry. Spleen cells from LDLR−/− BMT after 20 weeks of high fat diet were excised and pushed through a 70-µm strainer, and then red blood cells were lysed in RBC Lysis Buffer according to the manufacturer’s protocol (BioLegend). Cells were then incubated overnight with 10 µg/ml Dil-OxLDL (Biomedical Technologies) in Iftach’s media. Cells were then analyzed for Dil-OxLDL and stained for surface markers by flow as described above. For imaging of Dil-OxLDL uptake cells were grown on chamber slides, treated under similar conditions, and then were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Slides were imaged with a FluoView FV10i confocal laser-scanning microscope (Olympus).

Peritoneal macrophages were isolated by peritoneal lavage with or without thioglycolate stimulation as previously described. Cells were then cultured for either 1, 2, 4, 18 or 24 hours in Iftach’s media with or without 30 ng/ml LPS (Sigma, L2018), 30 µg/ml KLA (Avanti Polar Lipids), 5 µg/ml R848 (InvivoGen), or 30 ng/ml PAM2 (InvivoGen). Cell culture media was then collected for cytokine and nitric oxide analysis (described below). Cells were either lysed and frozen at -80 °C for RNA analysis (described below), or stained for surface marker and cytokine expression by flow cytometry.
**Bone marrow macrophage Dil-OxLDL staining.** Bone marrow cells were prepared from femurs and tibiae of 8- to 12-week-old mice as previously described \(^4,12,13\), with minor modifications. Surgically removed cleaned bones were left in 70% ethanol for 2–5 min for disinfection and washed with PBS, and the marrow was flushed with PBS in a syringe with a 0.45 mm diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting. Cells were seeded in 25 ml flasks with Ifach’s media (see above) and 30 ng/ml recombinant murine granulocyte macrophage-stimulating factor, rmGM-CSF (PeproTech) and cultured for 6 days. Cells were incubated with 10 μg/ml Dil-Ox-LDL (Biomedical Technologies, Stoughton, MA) for 6 hours and uptake was determined by flow cytometry.

**Quantitative real-time PCR.** Monocyte or macrophage populations were isolated by flow cytometry and total cellular RNA was collected with an RNeasy Plus Micro Kit according to the manufacturer’s protocol (Qiagen). RNA purity and quantity was measured with a nanodrop spectrophotometer (Thermo Scientific). Approximately 800 ng RNA was used for synthesis of cDNA with an Iscript cDNA Synthesis Kit (Bio-Rad). Total cDNA was diluted 1:20 in H₂O, and a volume of 9 μl was used for each real-time condition with a MyIQ Single-Color Real-Time PCR Detection System (Bio-Rad) and TaqMan Gene Expression Mastermix and TaqMan primers (see Online Table II for primer sequences). Data were analyzed and presented on the basis of the relative expression method. The formula for this calculation was as follows: relative expression = 2^(-ΔΔCT), where ΔΔCT is the change in cycling threshold between the gene of interest and the ‘housekeeping’ gene Gapdh (encoding glyceraldehyde 3-phosphate dehydrogenase), S is the result obtained with Nur77⁻/⁻ cells, and C is the result obtained with C57BL/6J control cells. The expression of each wild-type transcript was calculated relative to the mean wild-type transcript expression to show variability in wild-type samples. The mean wild-type transcript expression was then compared with that of transcripts from each Nur77⁻/⁻ sample to determine the percent change relative to wild-type expression.

**Inflammatory cytokine and nitric oxide measurements.** Media was collected from cultured peritoneal cells and analyzed for concentrations of interleukin-12p70 (IL-12), interleukin-10 (IL-10), and TNFα by ELISA according to manufacturer’s instructions (eBiosciences). Nitric oxide levels in media were measured by the Griess Reagent System according to manufacturer’s instructions (Promega).

**Human sample collection and analysis of Nur77 expression.** Peripheral blood collected from healthy donors, and monocytes purified by Ficoll gradient (Histopaque, Sigma) followed by red blood cell lysis and subsequent negative isolation with a kit (Stemcell Technologies which deplete CD2, CD3, CD19, CD20, CD56, CD66b, CD123 positive cells). Monocytes subtypes were isolated by cell sorting using anti-human CD14-APC and CD16-FITC antibodies.

**Statistical analysis.** Data for all experiments were analyzed with Prism software (GraphPad). Unpaired Student’s t-tests, Mann-Whitney, and two-way analysis of variance were used for comparison of experimental groups. P values of less than 0.05 were considered significant.

**References for Methods**

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Online Table I. Antibody clones used for flow cytometry.
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Online Table II. Quantitative PCR primers.
Online Figure I. Lipid profiles of ApoE−/− Nur77−/− mice on high fat diet. (A) Quantification of cholesterol content in blood serum from ApoE−/− Nur77−/− (Nur77−/−) and ApoE−/− (WT) mice by FPLC after 11 weeks of Western diet. (B) Quantification of cholesterol and triglyceride content in blood serum from ApoE−/− Nur77−/− (Nur77−/−) and ApoE−/− (WT) mice by FPLC after 11 weeks of Western diet (P=.07). (n = 8 per group; mean and s.e.m.).
Online Figure II. Specific reduction of Ly6C$^-$ monocytes in blood of Nur77$^{-/-}$ApoE$^{-/-}$ mice on high fat diet. (A) T cells, B cells, Granulocytes, NKT cells, Total monocyte (All mono), Ly6C$^+$ and Ly6C$^-$ monocyte populations in blood from Nur77$^{-/-}$ApoE$^{-/-}$ mice after 11 weeks of Western diet feeding examined by flow cytometry. Cell populations are expressed as percentage of all live cells in blood. (B) White blood cell, Neutrophil, Lymphocyte, Monocyte, Eosinophil and Basophil populations in blood from Nur77$^{-/-}$ApoE$^{-/-}$ mice after 11 weeks of Western diet feeding analyzed by Hemavet. Cell populations are expressed as thousand cells per microliter (K/$\mu$L) of blood. *P < 0.01 (unpaired Student’s t-test). n = 8 mice per group for both panels.)
Online Figure III. Total number of Nur77-deficient resident and thioglycolate-elicited peritoneal macrophages are similar to control. Resident peritoneal macrophages CD11b^+F4/80^+ from wild-type (WT) and Nur77^{-/-} mice were collected without thioglycolate (UN) or after thioglycolate (Thio) treatment. Thioglycolate (4% solution) was administered by peritoneal injection 5 days before macrophage collection. Cells were counted on a Coulter counter and percentage of macrophages were measured by flow cytometry. The percentage of macrophages cells was multiplied by cell counts to calculate total macrophage numbers. (n=6 mice per group)
Online Figure IV. Increased expression of NR4A family members with KLA activation. Induction of NR4A family members Nur77, NOR1 and Nurr1 in wild-type peritoneal macrophages in response KLA (100 ng/ml) stimulation for 2 hours measured by quantitative Real-Time PCR. Quantification of data expressed as percent change in KLA stimulated macrophage mRNA over untreated expression for each gene. (*P < 0.01 (unpaired Student’s t-test, n =6 mice per group).
Online Figure V. Gating strategy for identification of peritoneal macrophages. Single, live cells are gated for Lin\(^{-}\) (CD19, CD3\(\varepsilon\)), CD11b\(^{+}\) expression. Dendritic cells and eosinophils are gated out using CD11c\(^{+}\) and Siglec-F\(^{-}\) expression respectively. Remaining cells are gated on F4/80\(^{hi}\), CD11b\(^{hi}\) expression (large macrophages) and F4/80\(^{mid}\), CD11b\(^{mid}\) expression (small macrophages). Note, thioglycolate intraperitoneal injection elicits an influx of small macrophages believed to be derived from Ly6C\(^{+}\) monocytes.
Online Figure VI. Alteration of resident macrophage population composition in ApoE−/−Nur77−/− fed a Western diet.
Representative flow cytometry scatter-plot of peritoneal large (F4/80hi, CD11bhi) and small (F4/80mid, CD11bmid) macrophage populations in ApoE−/− Nur77−/− and ApoE−/− (WT) mice after 11 weeks of Western diet feeding. Numbers next to gates show percentages of cell population in plot. Quantification of total, large, and small macrophage populations expressed as a percentage of all live cells in the peritoneal cavity (right). *P < 0.01 (unpaired Student’s t-test). Data are representative of 3 independent experiments (n = 6 per group; mean and s.e.m.).