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The Nurr7 agonist Cytosporone B differentially regulates inflammatory responses in human polarized macrophages

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ABSTRACT

The orphan nuclear receptor Nur77 is involved in diverse cellular processes such as inflammation, proliferation, differentiation and survival. Stimuli like lipopolysaccharide (LPS) and tumor necrosis factor (TNF) increase Nur77 expression in human and murine macrophages, and it has been proposed that Nur77 plays a major role in dampening the inflammatory response. Here, we evaluated the expression and function of Nur77 in human anti-inflammatory and pro-inflammatory macrophages derived from blood monocytes cultured with macrophage colony-stimulating factor (M-MDMs) or granulocyte/macrophage colony-stimulating factor (GM-MDMs), respectively. Nur77 mRNA expression was significantly enhanced in M-MDMs compared with GM-MDMs, both constitutively and upon exposure to Toll-like receptor (TLR)2, 3, and 4 ligands. Nur77 activation with the agonist Cytosporone B (CsnB) significantly suppressed the production of TNF, interleukin (IL)-1β, IL-6, and IL-8 in GM-MDMs stimulated with LPS. In contrast, it tended to enhance the production of the anti-inflammatory cytokine IL-10. This effect was associated with reduced NF-κB p65 nuclear translocation. Similarly, Nur77 knockdown enhanced *TNF* production in GM-MDMs. CsnB effectively stimulated the transactivation activity of Nur77 in M-MDMs, but it did not alter cytokine synthesis or p65 nuclear translocation. However, Nur77 seemed to have a role in maintaining the anti-inflammatory profile of M-MDMs, since Nur77-deficient M-MDMs constitutively produced higher levels of *TNF* transcripts. Thus, in the absence of exogenous agonists, Nur77 activity favors the anti-inflammatory function of M-MDMs, whereas agonistic activation of this receptor preferentially drives attenuation of inflammation in inflammatory macrophages.

1. Introduction

The orphan nuclear receptor NR4A1/Nur77 is an immediate-early response gene belonging to the NR4A transcription factor subfamily that also comprises Nurr1 and NOR1 receptors. Nur77 expression is elevated in tissues like the central nervous system, pancreas, skeletal muscle, liver, and adipose tissue, where it regulates the expression of enzymes involved in glucose and lipid metabolism (Maxwell and Muscat, 2006; Zhao and Bruemmer, 2010). An array of stimuli rapidly and transiently induces its expression: growth factors (e.g., platelet-derived growth factor), neurotransmitters (e.g., histamine, serotonin) (Martínez-González and Badimon, 2005; Zhao et al., 2011), and

Abbreviations: ActD, Actinomycin D; CsnB, cytosporone B; GM-MDM, macrophages generated with GM-CSF; IKB, inhibitor of NF-κB; IKK, IκB kinase; LBD, ligand binding domain; M-MDM, macrophages generated with M-CSF; oxLDL, oxidized low-density lipoprotein; PGN, peptidoglycan; poly(I:C), polyinosinic-polycytidylic acid; TRAF, TNF receptor-associated factor.

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inflammatory factors such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), and oxidized low-density lipoprotein (oxLDL) (Pei et al., 2005; Shao et al., 2010). The rapid induction of Nur77 upon stimulation with inflammatory factors suggests that this receptor is required to regulate inflammatory responses. However, controversial findings have been reported about the role played by Nur77 in these settings. For instance, Nur77 promotes inflammation and nuclear factor (NF)-κ B activity through induction of NF-κB-inducing kinase (NIK) and inhibitor of NF-κB (IκB) kinase i (IKKi) gene expression in a RAW264.7 macrophage cell line over-expressing Nur77 (Pei et al., 2006). Besides, *NR4A1* ablation reduced NF-κB p65 activation in microglial cells following cerebral ischemic damage (Zhang et al., 2019). In contrast, several studies have also demonstrated that Nur77 suppresses NF-κB and activator protein-1 (AP-1) signaling pathways through transactivation and transrepression mechanisms. Nur77 over-expression induces IκBα transcription in vascular endothelial cells and inhibits NF-κB nuclear translocation; moreover, it blocks p65-mediated transcription by direct binding in LPS-stimulated RAW264.7 cells (You et al., 2009; Li et al., 2015a). Nur77 also acts as a negative regulator of IKK activation by impeding TNF receptor-associated factor (TRAF)6 activation. This has been demonstrated in several cell models, such as human (THP-1) and murine (RAW264.7) macrophage cell lines, mouse peritoneal macrophages, as well as different tissues from wild-type and Nur77$^{-/-}$ mice with experimental colitis or sepsis (Li et al., 2016; Wu et al., 2016). Further, Nur77 binds c-Jun and inhibits AP-1-dependent c-Jun promoter activity in vascular endothelial cells under basal and thrombin-stimulated conditions, leading to a decreased expression of c-Jun (Qin et al., 2014). Additional mechanisms of Nur77 anti-inflammatory signaling are metabolic reprogramming of macrophages (Koenis et al., 2018), as well as inhibition of inflammasome activation through repression of interferon regulatory factor (IRF) 1 expression (Ding et al., 2021).

The contribution of Nur77 to the outcome of inflammatory responses could be the consequence of tissue environment, cell, or stimuli-specific effects (Rodríguez-Calvo et al., 2017). In humans, Nur77 is expressed in macrophages within atherosclerotic lesions (Pei et al., 2005; Bonta et al., 2006) and in synoviocytes from patients with rheumatoid arthritis (McMorrow et al., 2013), but is down-regulated in colonic mucosal tissues of patients with inflammatory bowel diseases relative to those from healthy subjects (Wu et al., 2016). Studies on Nur77-deficient mice point to an anti-inflammatory role of this receptor in healthy and diseased animals. Elderly Nur77$^{-/-}$ mice spontaneously develop systemic inflammation (Li et al., 2015b). Nur77$^{-/-}$ mice also develop more severe diseases than their wild-type littermates in atherosclerosis, endotoxic shock, and colitis models (Hanna et al., 2012; Hamers et al., 2015; Li et al., 2016; Wu et al., 2016). Disease severity is concomitant with increased levels of pro-inflammatory factors such as TNF, IL-1β, IL-6, or nitric oxide (Li et al., 2015a).

Macrophages are critical players in the initiation and resolution of inflammatory responses. Environmental signals drive macrophage differentiation towards a variety of functional phenotypes, where M1 (classical) and M2 (alternative) macrophages represent the endpoints of the activation spectrum (Gordon and Taylor, 2005; Martinez et al., 2008). M1 are cytotoxic macrophages that produce inflammatory mediators and participate in host defense against intracellular pathogens and transformed cells, whereas M2 macrophages secrete anti-inflammatory cytokines and growth factors and are engaged in anti-inflammatory responses, angiogenesis, scavenging, and tissue remodeling (Gordon and Martinez, 2010; Locati et al., 2013; Vogel et al., 2014). Prototypic polarizing stimuli are interferon (IFN)-γ, LPS, granulocyte/macrophage colony-stimulating factor (GM-CSF) (M1), and IL-4, immune complexes, IL-10, or macrophage colony-stimulating factor (M-CSF) (M2) (Fleetwood et al., 2007; Locati et al., 2013; Schilling et al., 2018). Macrophage polarization is controlled by several transcription factors such as NF-κB, peroxisome proliferator-activated receptors, Kruppel-like factors, CCAAT/enhancer-binding proteins, signal transducers and activators of transcription, or IRFs (Sica and Mantovani, 2012). The contribution of NR4A receptors to macrophage polarization is still poorly understood; however, Nur77 deficiency promotes inflammatory phenotypes in murine macrophages (Hanna et al., 2012). In the present study, we evaluated the expression and activity of Nur77 in primary human pro- and anti-inflammatory macrophages for the first time. Our results suggest that Nur77 regulates the inflammatory responses elicited by these macrophage subsets.

2. Materials and methods

2.1. Media and reagents

Monocytes were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine (Hyclone Laboratories), and 1 % penicillin/streptomycin (Gibco). The following reagents were used: recombinant human GM-CSF (1000 U/ml) and M-CSF (10 ng/ml) were obtained from ImmunoTools and from Miltenyi Biotec, respectively; Cytosporone B (CsnB, 1 μg/ml), lipopolysaccharide (LPS, *Escherichia coli* 0111:B4, 100 ng/ml), Actinomycin D (ActD, 5 μg/ml), p38 mitogen-activated protein kinase (MAPK) inhibitor BIRB 796 (0.1 μM), MAPK kinase 1/2 (MEK 1/2) inhibitor UO126 (2.5 μM), c-jun *N*-terminal kinase (JNK) inhibitor SP600125 (30 μM), and NF-κB inhibitor BAY-117082 (10 μM), were from Sigma Aldrich; peptidoglycan (PGN, *Staphylococcus aureus*, 10 μg/ml) and polyinosinic:polycytidylic acid [poly(I:C), 50 μg/ml] were purchased from InvivoGen.

2.2. Monocyte subset isolation and differentiation into macrophages

Human peripheral blood mononuclear cells were obtained by Ficoll density gradient (Nycomed Pharma) from buffy coats of healthy donors. Blood samples were collected with the approval of the Blood Bank's Institutional Review Board at Centro Médico Nacional "La Raza" (IMSS, Mexico City) in compliance with the Declaration of Helsinki. CD14^{+} monocytes were purified by positive selection using magnetic separation systems (Miltenyi Biotec), as previously reported (González-Domínguez et al., 2016). Cells were cultured at 0.5–0.7 × 10^6 cells/ml for 6 days in a complete medium containing GM-CSF or M-CSF to generate GM-MDMs and M-MDMs, respectively. The cultures were fed with cytokines every-two days.

Six-day differentiated macrophages were incubated for 0.5–18 h with LPS, PGN, or poly(I:C) in the presence of CsnB or vehicle (DMSO) as a control. Additionally, macrophages were treated with CsnB or DMSO alone. Cells stimulated with LPS were also treated with MAPK and NF-κB inhibitors that were added 1 h before the stimulus. Culture supernatants from cells stimulated for 18 h with TLR agonists were harvested, and production of TNF, IL-1 β, IL-6, IL-8 and IL-10 was quantified with ELISA kits (BD Biosciences).

2.3. Flow cytometry assays

Phenotypic analyses were conducted in fully differentiated macrophages cultured for 18 h with CsnB or DMSO as a control. Indirect immunofluorescence was carried out with a primary monoclonal antibody directed against human CD36 (BD Biosciences) or with an isotype-matched control antibody, followed by incubation with FITC-labeled goat anti-mouse immunoglobulins (Ig). Staining was performed in the presence of 50 μg/ml of human IgG. Data were acquired on a FACSCalibur (BD Biosciences).

2.4. Western blot

Monocyte and macrophage lysates were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). Unspecific binding was blocked with 5 % nonfat dry milk. Membranes were incubated with mouse monoclonal antibodies to Nur77, glyceraldehyde-

3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology), γ-tubulin (Sigma Aldrich), or with a rabbit monoclonal antibody to IκBα (Cell Signaling Technologies). Then, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (Dako). Bands were detected using enhanced chemiluminescence (Amersham Biosciences).

2.5. Quantitative real-time polymerase chain reaction

RNA from CD14^{+} monocytes and macrophages differentiated for 1, 3, 5, or 6 days was obtained, and gene expression was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted using the RNeasy kit (Qiagen) and retrotranscribed with the RevertAid H Minus First Strand cDNA Synthesis kit (ThermoFisher Scientific). For qRT-PCR, mRNA levels of selected genes were detected using TaqMan® Gene Expression Assays (Applied Biosystem Inc.): Hs00374226_m1 (*NR4A1*), Hs00174128_m1 (*TNF*), Hs00174097_m1 (*IL1B*), Hs00174131_m1 (*IL6*), Hs00174103_m1 (*IL8*), Hs00961622_m1 (*IL10*), Hs01081598_m1 (*INHBA*), Hs01077958_s1 (*IFNB1*), Hs00234140_m1 (*CCL2*), Hs00169627_m1 (*CD36*), Hs00234007_m1 (*MSR1*), and Hs00355671_g1 (*NFKBIA*). Results were normalized relative to the expression of TATA-box binding protein (Hs00427620_m1, *TBP*) mRNA and expressed using the Δ Δ cycle threshold method. Gene expression was quantified using a 7500 Real-Time PCR System (Applied Biosystems Inc.).

2.6. mRNA stability assays

NR4A1 mRNA was quantified relative to *TBP* mRNA at various time points after adding ActD to the cell culture medium. RNA was extracted, and mRNA species were detected by qRT-PCR, as described above. *NR4A1* mRNA half-life ($t_{1/2}$) was calculated by linear regression analysis.

2.7. Macrophage transfection with small interfering RNA for Nur77

Macrophages were transfected with Nur77-specific small interfering RNA (siNur77, 100 nM, sc-36109, Santa Cruz Biotechnology) using HiperFect® transfection reagent (Qiagen). Control cells were transfected with scrambled siRNA (siCtrl, 100 nM, control oligonucleotide AM4637, Ambion®, ThermoFisher Scientific). The transfection reagent was mixed with the siRNA, and this mixture was added to four-day differentiated macrophages. After 48 h, cells were harvested and analyzed as indicated.

2.8. Confocal microscopy

Macrophages were pre-treated with CsnB or DMSO for 2 h and then left unstimulated or exposed to LPS for 1 h. Subsequently, cells were fixed, permeabilized, and incubated with a primary antibody to NF-κB p65 (Cell Signaling) or with an isotype-matched control antibody in the presence of 50 μg/ml of human IgG. Then, cells were stained with Cy™5-labeled donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories). The nuclei and cytoskeletons of the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and rhodamine-conjugated phalloidin, respectively. The images were acquired on an SP8 Confocal microscope (Leica Microsystems). Quantification of protein expression [as relative fluorescence intensity (RFI) within regions of interest] was performed in three random fields (X20)/ sample with the FIJI software (ImageJ, National Institutes of Health) using similar acquisition settings in all samples.

2.9. Statistical analyses

Values are given as mean ± standard error of the mean (SEM) for separate experiments. Statistical analysis was carried out using the paired or unpaired two-tailed Student's *t*-test for the comparisons between two groups of data. A p-value < 0.05 was considered significant. Data were plotted using the Prism 5.0 software (GraphPad Software Inc.).

3. Results

3.1. Pro- and anti-inflammatory macrophages expressed Nur77

We previously evaluated the transcriptomic profile associated with the human macrophage lineage and polarization through gene expression microarray assays with GM-MDMs and M-MDMs (GEO accession no. GSE68061; http://www.ncbi.nlm.nih.gov/geo/) (González-Domínguez et al., 2016). We detected that the gene expression of the transcription factor Nur77 (encoded by the *NR4A1* gene) was higher in M-MDMs derived from CD14^{+}CD16^{-} monocytes than in GM-MDMs. These data were confirmed by qRT-PCR using total RNA extracted from additional donors, with an average increment of 2-fold (Fig. 1A). The higher expression level of *NR4A1* in M-MDMs seems to be caused by an enhanced transcription rate and not by an extended mRNA half-life since *NR4A1* mRNA almost doubled its half-life in GM-MDMs (39.4 min) with respect to M-MDMs (22 min) (Fig. 1B). The expression of Nur77 at the protein level was variable between donors. Still, it was significantly higher in M-MDMs, and both macrophage subsets showed enhanced Nur77 expression with respect to their monocyte precursors (Fig. 1C). *NR4A1* expression was also evaluated in monocytes and macrophages after 1, 3, and 5 days of differentiation (Fig. 1D). CD14^{+} monocytes expressed high levels of *NR4A1*. In contrast, the amount of *NR4A1* transcripts dramatically decreased on the first day of culture (600–900-fold) in both macrophage subsets. From there, *NR4A1* expression increased along with the culture time in M-MDMs, whereas GM-MDMs maintained low levels of this transcript for the entire differentiation process. Nevertheless, Nur77 was not readily detectable in both macrophage subtypes at the protein level until the last days of the differentiation process (Fig. 1D).

3.2. Nur77 expression is induced by TLR activation

Nur77 expression is induced by various stimuli, including pathogen-associated molecular patterns (PAMPs) (Pei et al., 2005). To determine whether the higher constitutive expression of *NR4A1* in M-MDMs correlates with an enhanced induction by these stimuli, we investigated the *NR4A1* expression kinetics upon activation of macrophages with different TLR ligands: PGN (TLR2), poly(I:C) (TLR3), and LPS (TLR4) (Fig. 2A). The expression of *NR4A1* transcripts was rapidly induced in M-MDMs, peaking 1 h after TLR3 and TLR4 activation, and 2 h following TLR2 activation. Interestingly, *NR4A1* induction in GM-MDMs was delayed compared to M-MDMs, peaking 4 h after TLR activation. Once induction peaked, the amount of *NR4A1* transcripts rapidly declined and returned close to baseline levels in 2–3 h in both macrophage subtypes (Fig. 2A). LPS and poly(I:C) were the major inducers of *NR4A1* expression in GM-MDMs (~3 times higher than PGN). In contrast, the three PAMPs induced similar levels of *NR4A1* mRNA in M-MDMs, and only poly(I:C) induction was slightly smaller (by 1.5-fold) (Fig. 2A). Nevertheless, the fold induction of *NR4A1* at the peak of expression was consistently higher in M-MDMs than in GM-MDMs, with an average of 86-fold increase for PGN, 25-fold for LPS, and 6-fold for poly(I:C) (Fig. 2B).

It has been reported that NF-κB (Pei et al., 2005) and MAPK (Shao et al., 2010) pathways are involved in Nur77 induction. Thus, we evaluated the relative contribution of these signaling pathways to *NR4A1* expression in LPS-challenged macrophages. The amount of *NR4A1* mRNA was deeply reduced in M-MDMs by the NF-κB and p38 inhibitors. Only the former induced a significant reduction of *NR4A1* expression in GM-MDMs (Fig. 2C). ERK1/2 and JNK inhibitors did not have substantial effects. Therefore, *NR4A1* transcription is strongly

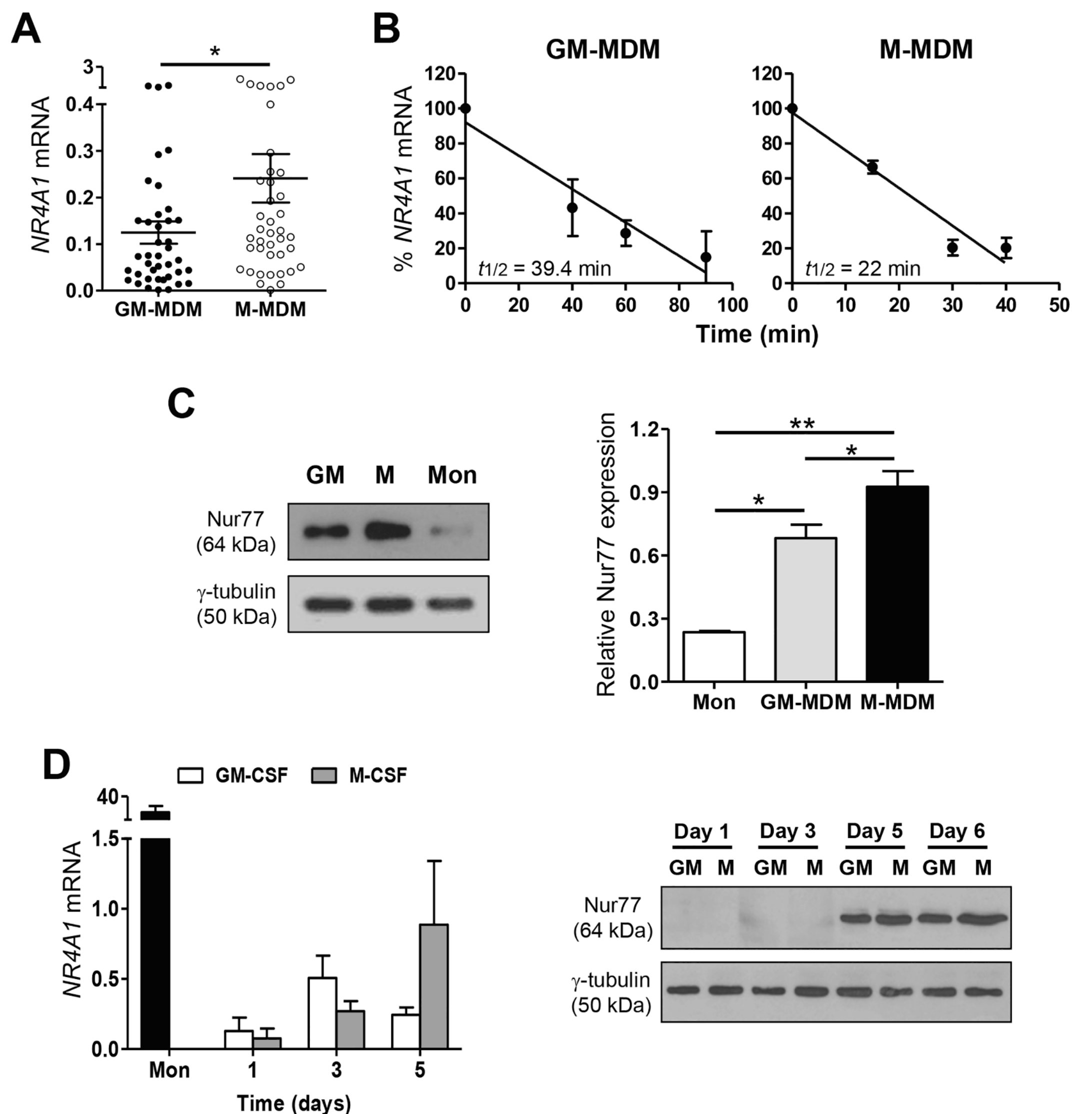

Fig. 1. Nur77 expression in primary macrophages generated *in vitro*. (A) *NR4A1* mRNA expression in fully differentiated GM-MDMs (black circles) and M-MDMs (empty circles) evaluated by qRT-PCR. Blood monocytes were cultured in the presence of GM-CSF or M-CSF for six days to generate GM-MDMs and M-MDMs, respectively. Each symbol represents the results of a single donor (n = 40; *, $p < 0.05$). (B) Analysis of NR4A1 mRNA stability in GM-MDMs (left) and M-MDMs (right). Cells were treated with the transcription inhibitor ActD, and total RNA was harvested at 15, 30, 40, 60, or 90 min post-treatment. *NR4*A1 mRNA levels were calculated relative to *TBP* mRNA by qRT-PCR. NR4A1 mRNA half-life ($t_{1/2}$) was determined by linear regression analysis (n = 3). (C) Western blot (left) and densitometric analysis (right) of Nur77 expression. Expression of Nur77 was evaluated in GM-MDMs (GM), M-MDMs (M), and CD14^{+} blood monocytes (Mon). Western blot quantification was normalized with the housekeeping proteins GAPDH or γ-tubulin. Mean ± SEM of 2 (monocytes) or 6 (macrophages) independent donors is shown (*, $p < 0.05$; **, $p < 0.005$). (D) Expression of Nur77 along the monocyte-to-macrophage differentiation process. Left: amount of *NR4A1* transcripts analyzed in freshly isolated blood monocytes (Mon, black bar) and in differentiating macrophages cultured for 1, 3 or 5 days with GM-CSF (white bars) or M-CSF (gray bars) (n = 4). Right: representative Western blot of Nur77 expression. In (A) and (D) the *NR4A1* mRNA level is depicted relative to *TBP* expression.

induced by the NF-κB and MAPK signaling pathways in M-MDMs exposed to LPS; however, NF-κB is the primary inducer of this gene in LPS-stimulated GM-MDMs.

At the protein level, Nur77 was rapidly induced (1 h) in M-MDMs upon LPS exposure. Its levels rose until the last time evaluated (8 h) (Fig. 2D). In agreement with that observed at the mRNA level, Nur77 induction following LPS stimulation was delayed in GM-MDMs. We could only detect an increase of Nur77 after 8 h of activation (Fig. 2D).

Altogether, our data indicate that Nur77 is highly and rapidly induced at the mRNA and protein levels in M-MDMs challenged with different PAMPs. However, this induction is of lesser magnitude and delayed in time in GM-MDMs.

3.3. The Nur77 agonist CsnB activates Nur77 transcriptional activity in pro- and anti-inflammatory macrophages

Previous studies have demonstrated that Nur77 limits inflammatory responses (You et al., 2009, Hamers et al., 2015, Li et al., 2016, Wu et al., 2016). To explore the potential role of Nur77 on the macrophage inflammatory profile, we evaluated the activity of the Nur77 agonist CsnB on inflammatory gene expression. Previous studies indicated that CsnB increases the transactivation activity of Nur77 by promoting its interaction with the steroid receptor coactivators SRC-1 and SRC-2 and through positive autoregulation of *NR4A1* mRNA levels (Zhan et al., 2008).

Treatment of both types of macrophages with CsnB for 2 h led to a significant increment of *NR4A1* gene expression (Fig. 3A), confirming that this agonist stimulates the transactivation activity of Nur77 in our

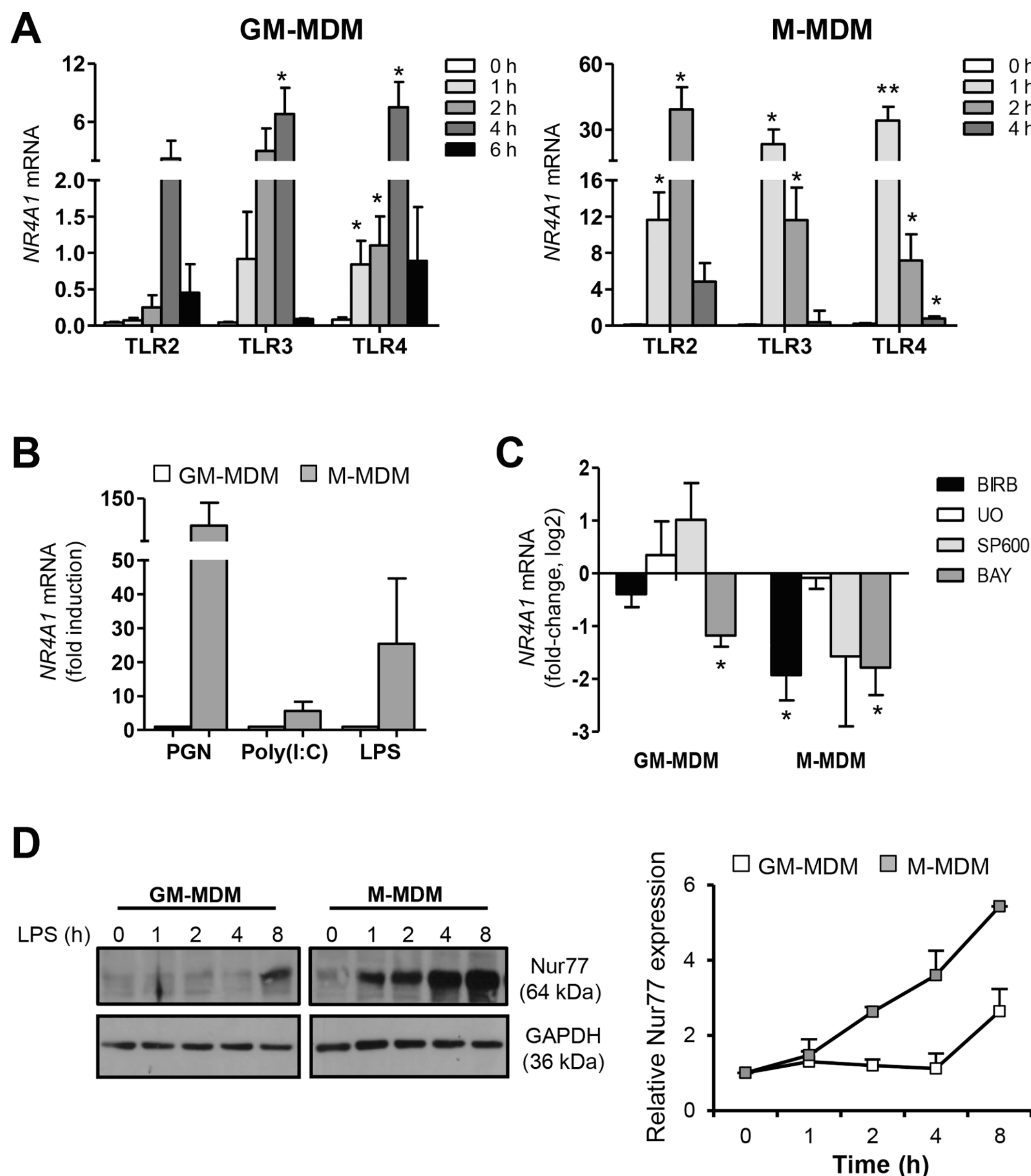

Fig. 2. TLR ligands up-regulate *NR4A1* mRNA expression in human macrophages. (A) Fully differentiated macrophages were stimulated with PGN, poly(I:C), or LPS at the indicated time points. *NR4A1* mRNA expression was evaluated by qRT-PCR in GM-MDMs (left) and M-MDMs (right). Data are depicted as the amount of *NR4A1* transcripts relative to *TBP* mRNA (n = 4–7). (B) Comparison of *NR4A1* mRNA levels between GM-MDMs and M-MDMs following TLR activation at the peak of induction (GM-MDMs 4 h, M-MDMs 1 h). Data are expressed as fold-induction in M-MDMs with respect to GM-MDMs. (C) Macrophages were stimulated with LPS in the presence of DMSO, p38 (BIRB), MEK 1/2 (UO), JNK (SP600), or NF-κB (BAY) inhibitors. The levels of *NR4A1* transcripts at the peak of induction (GM-MDM 4 h, M-MDM 1 h) were evaluated by qRT-PCR as in (A). Results are expressed relative to the levels of *NR4A1* mRNA in the presence of DMSO. The y-axis shows the $\log_2$-fold change between inhibitor and DMSO conditions (n = 3). (D) Detection of Nur77 in whole-cell lysates of unstimulated (0 h) and LPS-stimulated macrophages at the indicated time points. Western blot and densitometric analysis from two independent donors are shown. Quantification was normalized with the housekeeping protein GAPDH and is shown relative to unstimulated cells. Statistics: LPS-activated *vs* unstimulated cells (A), DMSO- *vs* inhibitor-treated cells (C). *, $p < 0.05$; **, $p < 0.005$.

cellular system. It has been shown that Nur77 over-expression reduces the transcription of modified lipoprotein receptor genes, such as *CD36* and *MSR1* (Bonta et al., 2006). In our study, Nur77 activation with CsnB also tended to down-regulate the gene expression of these receptors in GM-MDMs, though their expression was not modified in M-MDMs (Fig. 3A).

The effect of CsnB was assessed regarding the constitutive transcription of several cytokines/chemokines related to macrophage polarization: *TNF*, *IL1B*, *IL6*, *IL8*, *IL10*, *INHBA* (encoding activin A subunit inhibin β A), *IFNB1* (encoding interferon beta), and *CCL2* (encoding C—C motif ligand 2). As shown in Fig. 3B, the pro-inflammatory phenotype of GM-MDMs is associated with enhanced basal expression of *TNF*, *IL1B*, *IL6*, *IL8*, and *INHBA*, and reduced levels of *IL10* and *CCL2* mRNA compared to M-MDMs. Both GM-MDMs and M-MDMs treated with CsnB tended to reduce the mRNA levels of *IL1B* and *CCL2* and to enhance those of *INHBA* and *IFNB1* (Fig. 3C). Particularly, CsnB significantly down-regulated the levels of the transcripts encoding the chemokines IL-8 and CCL2 in GM-MDMs, while the amount of *IL6* and *IL1B* transcripts was significantly reduced in CsnB-treated M-MDMs (Fig. 3C). No significant changes were found among the remaining cytokines evaluated. Hence, increasing Nur77 activity did not seem to affect macrophage polarization status globally.

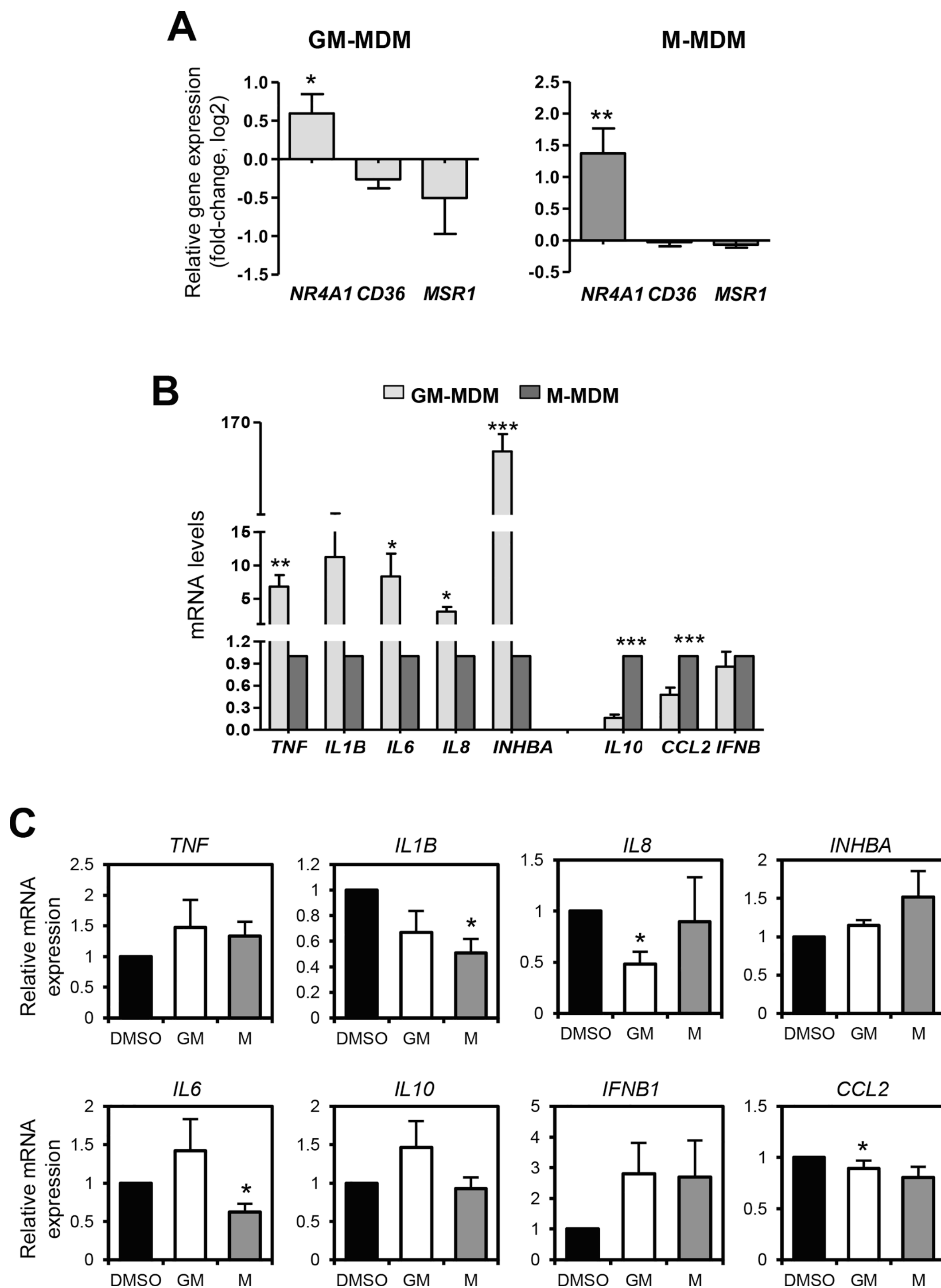

Fig. 3. The increased transactivational activity of Nur77 induced by CsnB leads to an altered profile of cytokine transcription. (A) GM-MDMs (left) and M-MDMs (right) were treated with the Nur77 agonist CsnB for 2 h or with DMSO as a control. *NR4A1, CD36,* and *MSR1* gene expression was evaluated by qRT-PCR. Results are expressed relative to the mRNA levels in the presence of DMSO (n = 5–11). The *y*-axis shows the $\log_2$-fold change between DMSO and CsnB conditions. (B) Constitutive expression of *TNF*, *IL1B*, *IL6*, *IL8*, *INHBA*, *IL10*, *CCL2*, and *IFNB1* transcripts in GM-MDMs relative to M-MDMs (n = 8–50). (C) Evaluation of *TNF*, *IL1B*, *IL6*, *IL8*, *INHBA*, *IFNB1*, *CCL2*, and *IL10* gene expression in GM-MDMs (GM) or M-MDMs (M) treated with CsnB for 2 h or with DMSO as a control. Results are expressed relative to the mRNA levels in the presence of DMSO (n = 4–13). Statistics: DMSO- *vs* CsnB-treated cells (A, C), GM-MDMs *vs* M-MDMs (B). *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

To investigate whether these changes were reflected at the protein level, macrophages were treated for 18 h with CsnB, and the concentration of cytokines was measured in the culture supernatants. GM-MDMs were the most affected cells by CsnB treatment. They significantly diminished their secretion of IL-6 (by 1.5-fold) and IL-8 (by 1.7-fold) and tended to reduce their TNF production (by 1.7-fold) (Fig. 4A). In contrast, the secretion of TNF, IL-1 β, IL-6, IL-8, and IL-10 remained unaffected in M-MDMs (Fig. 4A). The most significant effect of CsnB on GM-MDMs was also observed on the CD36 receptor expression. GM-MDMs reduced their levels of CD36 by 2-fold after 18 h of CsnB

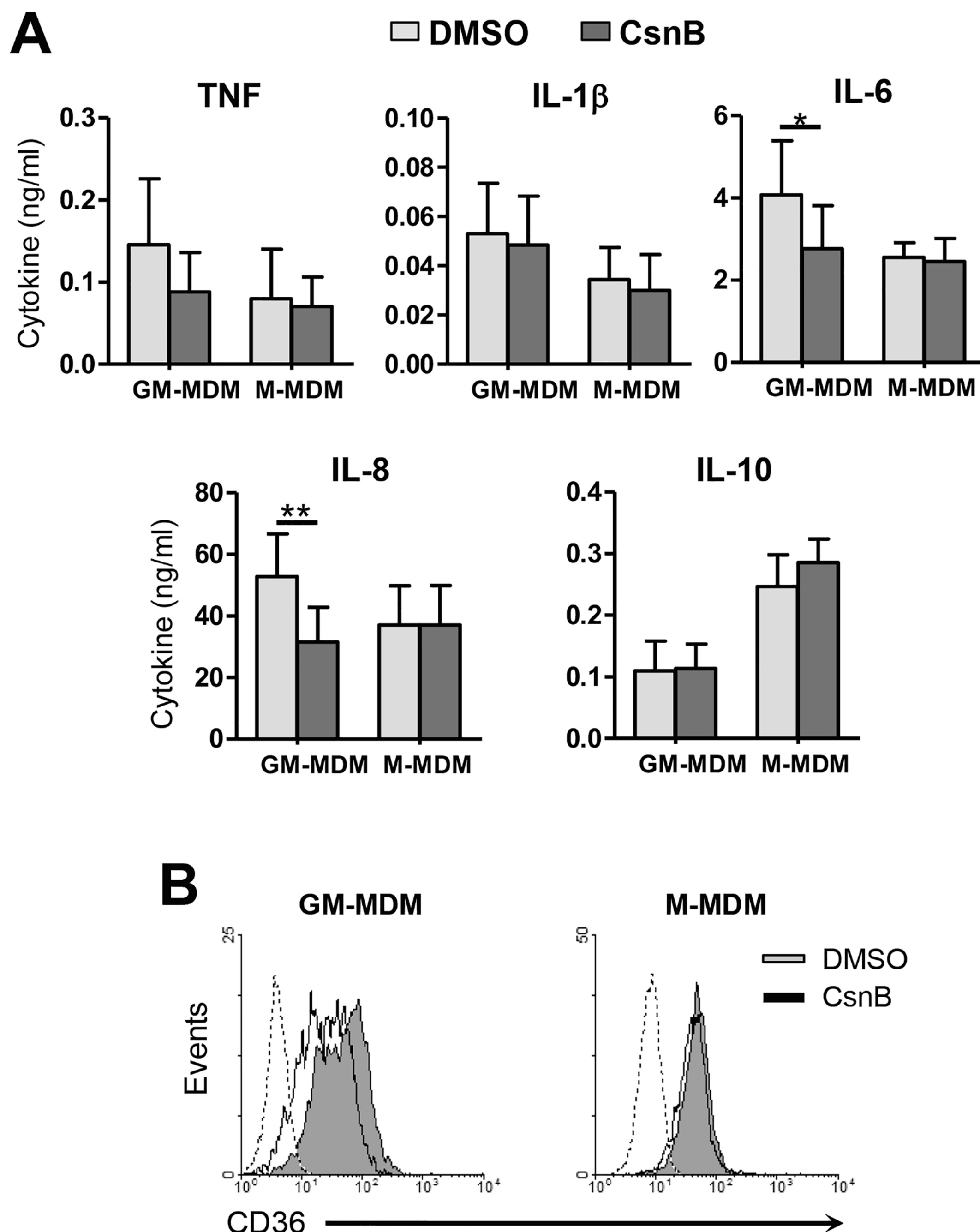

Fig. 4. Nur77 activation attenuates the pro-inflammatory profile of GM-MDMs. (A) GM-MDMs and M-MDMs were treated with the Nur77 agonist CsnB for 18 h, or with DMSO as a control. Production of the indicated cytokines/chemokines was evaluated by ELISA in the culture supernatants (n = 4–10; *, $p < 0.05$; ** $p < 0.005$). (B) GM-MDMs (left) and M-MDMs (right) cultured in the absence (DMSO, solid gray histogram) or presence of CsnB (empty black histogram) for 18 h were assessed for the expression of CD36 by flow cytometry. Empty dotted line histograms represent the staining with Ig isotype-matched control. Results are representative of 2 donors evaluated.

exposure, whereas no change was detected in M-MDMs (Fig. 4B).

Collectively, these data indicate that CsnB activates the transcriptional activity of Nur77 in pro- and anti-inflammatory macrophages. However, its effects on limiting inflammation appear to be greater in GM-MDMs than in M-MDMs.

3.4. Nur77 suppresses pro-inflammatory cytokine secretion induced by TLR activation in GM-MDMs

To further investigate whether Nur77 activation could modulate the inflammatory response of human macrophages activated with PAMPs, GM-MDMs and M-MDMs were stimulated with LPS in the presence of CsnB. Gene expression was evaluated at 2 and 12 h after LPS activation. CsnB-treated GM-MDMs tended to down-regulate the expression of the pro-inflammatory cytokines *TNF*, *IL6*, *IL8*, and *CCL2* (only significant for *IL8*), while they tended to up-regulate the transcription of *IFNB1* and *IL10* (Fig. 5A). Nevertheless, most of the changes were subtle. Regarding M-MDMs, we could not detect a clear pro-inflammatory or anti-inflammatory cytokine profile induced by CsnB. At early time points of LPS exposure, Nur77 activation promoted a slight but significant increment of *IL1B* mRNA levels (Fig. 5A). However, at late time points, it tended to down-regulate its expression. Analogous to that found in GM-

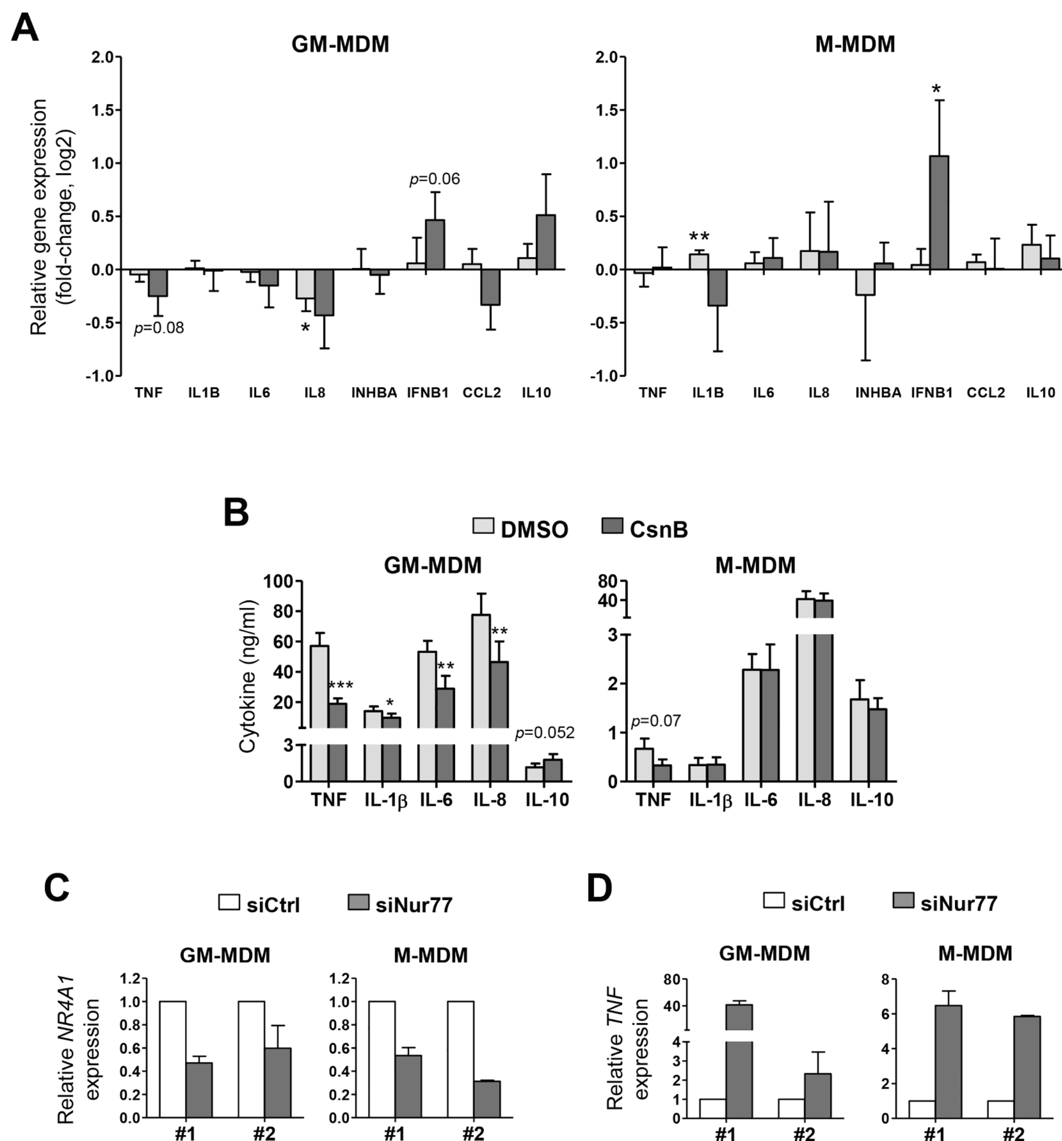

Fig. 5. Nur77 suppresses pro-inflammatory cytokine production upon TLR4 activation. (A) Relative gene expression (log_2-transformed fold-change) for the indicated cytokines/chemokines in GM-MDMs (left) and M-MDMs (right) stimulated for 2 or 12 h with LPS, in the presence of CsnB or vehicle (DMSO). Results are expressed relative to the levels of each gene in the presence of DMSO, as in Fig. 3A (n = 4–7; *, $p < 0.05$). (B) Production of the indicated cytokines/chemokines in GM-MDMs (left) and M-MDMs (right) stimulated with LPS for 18 h, in the presence of CsnB or vehicle (DMSO), evaluated by ELISA (n = 4–10; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$). (C, D) Relative expression of *NR4A1* (C) and *TNF* (D) mRNA in macrophages transfected with siCtrl or siNur77 generated from two independent donors (#1, #2). Results are shown as the expression of each gene in siNur77-treated cells relative to its expression in siCtrl-treated samples and represent the mean ± SEM of duplicates.

MDMs, CsnB favored the increase of *IFNB1* and *IL10* mRNA levels in M-MDMs (Fig. 5A). The generalized decrease of pro-inflammatory cytokine transcription found in GM-MDMs exposed to CsnB was corroborated at the protein level, with a significant reduction of TNF (by 3-fold), IL-6 and IL-8 (by almost 2-fold), and IL-1 β (by 1.4-fold) secretion after 18 h of exposure to LPS. Moreover, they increased the synthesis of IL-10 by 1.5-fold (Fig. 5B), concurring with the increment of *IL10* transcription. Conversely, CsnB did not modify the production of these cytokines in M-MDMs except for TNF, which tended to diminish its levels following CsnB exposure (Fig. 5B).

The differential regulation of cytokine production by Nur77 was not specific to LPS-driven responses since treatment of GM-MDMs with CsnB significantly attenuated TNF and IL-8 production induced by the TLR2 and TLR3 ligands PGN and poly(I:C), respectively (Supplementary Fig. 1A, B). The levels of IL-1 β also tended to diminish in the presence of CsnB, whereas IL-10 tended to be up-regulated (Supplementary Fig. 1A, B). Hence, these data demonstrate that the regulatory mechanisms coordinated by Nur77 are not limited to TLR4 signaling. Analogous to the results obtained for LPS, CsnB did not modify the cytokine secretion induced by PGN and poly(I:C) in M-MDMs.

To further substantiate the above data, we silenced *NR4A1* expression. Endogenous *NR4A1* mRNA levels were decreased by 40–70 % in macrophages transfected with a specific siRNA for Nur77 (siNur77) compared with control siRNA (siCtrl) (Fig. 5C). These siNur77 targeted macrophages, both GM-MDMs and M-MDMs, constitutively expressed increased levels of TNF mRNA with respect to siCtrl-treated cells (Fig. 5D). Therefore, our data indicate that Nur77 controls to a certain extent the inflammatory profile of primary human macrophages regardless of their polarization status.

3.5. *CsnB diminishes NF-κB nuclear translocation in GM-MDMs exposed to LPS*

Several studies have demonstrated that Nur77 suppresses NF-κB

signaling pathway (Hamers et al., 2015; Wu et al., 2016; Li et al., 2017), and some indicate that Nur77 can positively regulate IκBα expression by direct binding to a Nur77 response element in the IκBα promoter (You et al., 2009). Thus, we analyze whether the regulation of cytokine transcription by CsnB paralleled an induction of *NFKBIA*. Neither GM-MDMs nor M-MDMs altered *NFKBIA* transcription when Nur77 was activated by CsnB (Fig. 6A). However, CsnB prevented IκBα degradation in GM- and M-MDMs activated with LPS for 15 min (Fig. 6B, C). This finding leads us to evaluate NF-κB p65 nuclear translocation in macrophages in the presence of CsnB. The cells were pre-treated for 2 h with CsnB or DMSO as control. The subcellular localization of p65 was analyzed by Confocal microscopy in the steady state and after 1 h of LPS exposure. In the absence of TLR stimulation, CsnB slightly increased the nuclear localization of p65 in both GM- and M-MDMs (Fig. 6D). However, macrophage activation with LPS promoted a massive translocation of p65 to the nucleus that was reduced in GM-MDMs, but not in M-MDMs, in the presence of CsnB (Fig. 6D, E). This decrease was concomitant with an enhanced percentage of cytosolic p65 in GM-MDMs (Supplementary Fig. 2A). In contrast, this percentage did not vary or even tended to diminished in M-MDMs (Supplementary Fig. 2B). Therefore, despite CsnB inhibits IκBα degradation at the beginning of LPS activation in both macrophage subtypes, a decrease of NF-κB p65 nuclear localization is only achieved in GM-MDMs and could explain the preferential anti-inflammatory role of CsnB in these cells.

4. Discussion

The complex activity of Nur77 coordinating inflammatory responses is evidenced by its specific effects on gene expression in different cellular contexts. Functional studies reveal both pro- and anti-inflammatory roles for Nur77 (Pei et al., 2006, Shao et al., 2010, Maijenburg et al. 2012, Lappas 2014, Hamers et al., 2015). Here we demonstrate that Nur77 regulates the inflammatory response in human macrophages, and our results support the role of this receptor in the resolution of

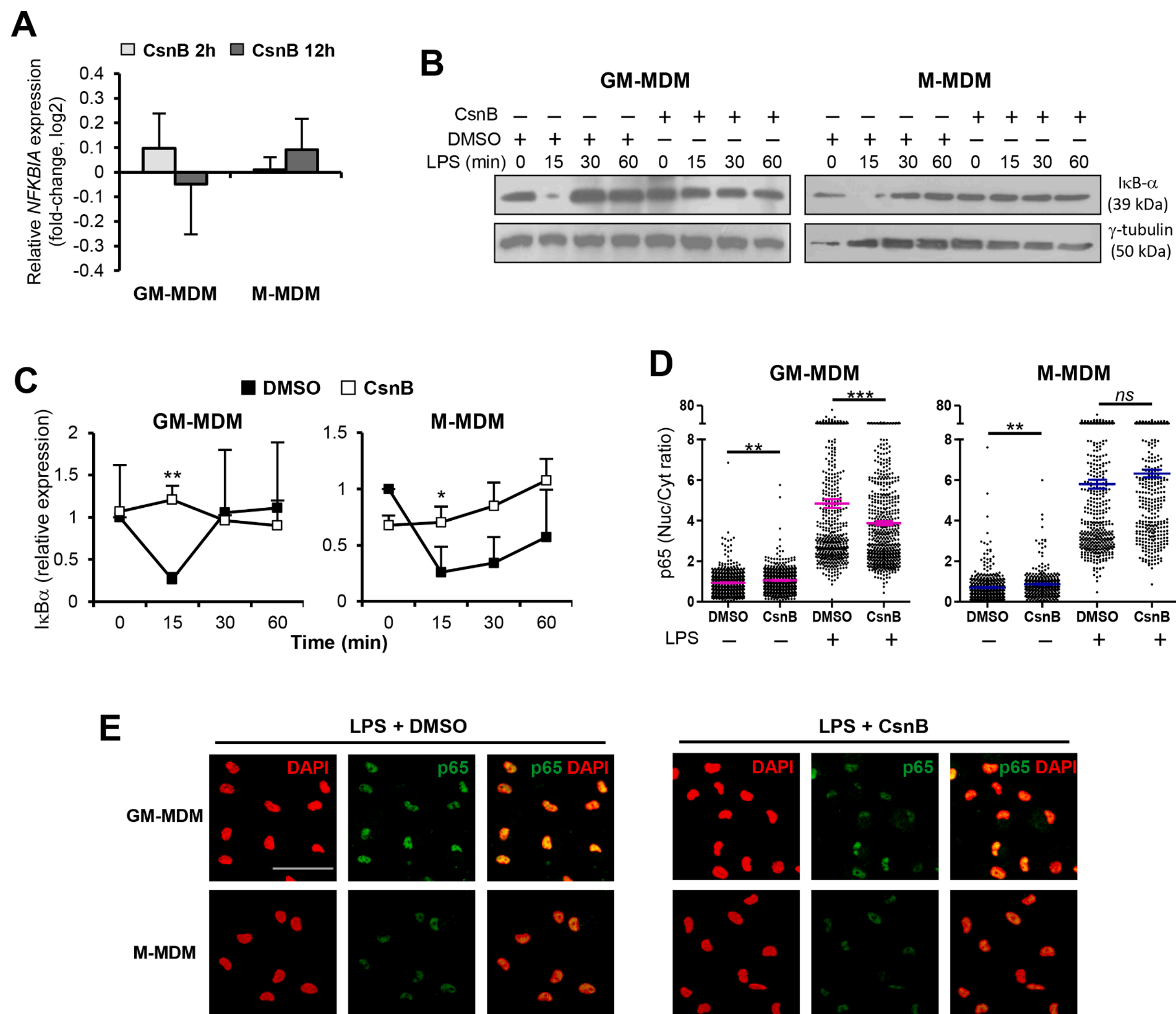

Fig. 6. CsnB reduces NF-κB nuclear translocation in LPS-activated GM-MDMs. (A) Expression of *NFKBIA* in GM-MDMs and M-MDMs stimulated for 2 or 12 h with LPS, in the presence of CsnB or vehicle (DMSO) (n = 3). Results are plotted as in Fig. 3A. (B, C) IκBα degradation in GM- and M-MDMs pre-treated for 2 h with CsnB or vehicle (DMSO), and then exposed to LPS for 15–60 min. A representative Western blot for IκBα expression in GM-MDMs and M-MDMs (B) and densitometric quantification of three independent donors (C) is shown. Values of IκBα expression in (C) were normalized to those of untreated cells cultured with DMSO (*, $p < 0.05$; **, $p < 0.005$). (D, E) Quantification of NF-κB p65 expression in macrophages left untreated or treated with LPS for 1 h, in the presence of CsnB or vehicle (DMSO), evaluated by Confocal microscopy. (D) Scatter plot showing the ratio of p65 RFI in the nuclear and cytoplasmic compartments of unstimulated (-) or LPS-activated (+) cells (1 h) in the absence (DMSO) or presence of CsnB. Each point represents a single cell data; 200–450 cells for each condition are depicted from 3 to 4 different donors (**, $p < 0.005$; ***, $p < 0.0005$; ns: not significant). (E) Representative micrographs of double immunofluorescence staining for p65 (green) and DAPI (red) in LPS-treated macrophages in the absence (DMSO) or presence of CsnB. Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inflammation.

We found that anti-inflammatory M-MDMs have higher constitutive and induced *NR4A1* gene expression with respect to pro-inflammatory GM-MDMs, which was not due to enhanced transcript half-life. Our results align with reports showing *NR4A1* expression as a feature of reparative, anti-inflammatory macrophages in mouse models (Ipseiz et al., 2014, Chen et al., 2022). It has been reported that persistent inflammatory stimulation results in a decline of Nur77 levels after an initial induction in rat chondrocytes dependent on epigenetic silencing (Xiong et al., 2020). Thus, one possible explanation for the low levels of *NR4A1* in GM-MDMs could be the chronic inflammatory signals that GM-CSF elicits along the culture.

Nur77 expression is induced by pro-inflammatory stimuli like LPS and TNF in THP-1 cells, primary human monocytes and murine macrophages (Pei et al., 2005), oxLDL in mouse macrophages (Shao et al., 2010), or PGF_2 in luteal cells (Stocco et al., 2002). Here we showed that the *NR4A1* gene was induced in pro- and anti-inflammatory macrophages upon activation with TLR2, TLR3, and TLR4 ligands, indicating that the *NR4A1* gene expression can be separately induced by MyD88- and TRIF-dependent mechanisms (Brubaker et al., 2015). Nur77 induction by LPS was mediated through the NF-κB and MAPK signaling pathways in M-MDMs, and the NF-κB pathway in GM-MDMs. In line with these results, the Nur77 promoter contains binding sites for both NF-κB and AP-1 transcription factors (Pei et al., 2005), and NF-κB and MAPK p38 contribute to Nur77 induction in macrophage cell lines (Pei et al., 2005, Shao et al., 2010). However, Nur77 mRNA and protein accumulate with different kinetics in GM-MDMs and M-MDMs upon TLR stimulation. In both GM-MDMs and M-MDMs there was a transient induction and a rapid messenger drop, concurring with the profile of immediate-early response genes (Bahrami and Drabløs, 2016), but with a sustained protein induction that was particularly strong in M-MDMs. Upon stimulation, the expression of *NR4A1* mRNA was also higher and peaked earlier in M-MDMs than in GM-MDMs, which could account for the significant amounts of Nur77 synthesized in M-MDMs exposed to LPS.

The Nur77 agonist CsnB is a fungal metabolite that stimulates Nur77-dependent transactivation activity and up-regulates its transcription through distal consensus NGFI-B response elements (NBRE) at the *NR4A1* promoter (Zhan et al., 2008). Moreover, CsnB increased the levels of Nur77 in the liver and colon of mice exposed to this agonist (Hu et al., 2014, Wu et al., 2016). Consistent with these reports, our data reveal that CsnB was able to induce *NR4A1* transcription in both anti- and pro-inflammatory macrophages. Consequently, it can be inferred that this compound activates Nur77 transcriptional activity in both cell types. However, the higher expression of Nur77 in M-MDMs did not correlate with their ability to prevent the synthesis of inflammatory mediators in the presence of CsnB, and we evidenced that this role was almost exclusively associated with GM-MDM function. CsnB ameliorated the constitutive secretion of IL-6 and IL-8 in GM-MDMs and diminished their basal expression of CD36. The effects of CsnB were more drastic when GM-MDMs were exposed to LPS, with a profound decrease in the secretion of all pro-inflammatory cytokines evaluated (TNF, IL-1 β, IL-6, IL-8). IL-8 was one of the most down-regulated cytokines in CsnB-treated GM-MDMs, which could be relevant *in vivo* to reduce the influx of granulocytes to inflamed tissues. In line with this result, colonic explants from Nur77-deficient mice with experimental colitis secreted increased levels of the KC chemokine (the mouse orthologue of IL-8) compared to those of wild-type mice, which was associated with higher neutrophil infiltration in the colons of $Nur77^{-/-}$ mice (Hamers et al., 2015). Our results agree with studies reflecting the anti-inflammatory properties of CsnB. This compound reduced the constitutive gene expression of *TNF*, *IL1B*, *IL6*, *IL12*, *CRP*, and *ICAM1* in THP-1 macrophages (Hu et al., 2014), as well as the expression of LPS-induced *Tnf*, *Il1b*, *Nos2*, and *Ptgs2* in BV2 microglia (Liu et al., 2017). Moreover, CsnB decreased CD36 expression in THP-1 macrophage-derived foam cells and in CsnB-treated $ApoE^{-/-}$ mice, which paralleled a decreased of oxLDL uptake and cellular cholesterol content (Hu et al., 2014). Consequently, Nur77 activation inhibits plaque progression in animal models of atherosclerosis. Additionally, CsnB decreased TNF and IL-6 levels in serum from wild-type mice with experimental colitis, while they were not affected in Nur77-deficient mice (Wu et al., 2016). These examples underscore the critical effects of Nur77 in inflammatory diseases and the valuable therapeutic use of Nur77 agonists.

Nur77 modulates inflammatory responses via transrepression and transactivation mechanisms. The anti-inflammatory state generated by Nur77 activation has been mainly attributed to the attenuation of NF-κB signaling (You et al., 2009, Hamers et al., 2015, Li et al., 2015a, Wu et al., 2016). At the transcriptional level, Nur77 over-expression activates the transcription of *NFKBIA* in endothelial cells (You et al., 2009), thus promoting IκBα expression. Nevertheless, *NFKBIA* gene expression was not modulated by CsnB in our cellular system. Instead, CsnB did prevent IκBα degradation at early time points of LPS stimulation and reduced p65 nuclear translocation in GM-MDMs, in line with previous results (Liu et al., 2017, Yan et al., 2020). Silencing IκBα expression blocked the anti-inflammatory effects of CsnB in a Parkinson's cell model, where this agonist inhibited IκBα phosphorylation (Yan et al., 2020). Thus, inhibition of IκBα phosphorylation by the IκB kinase complex and its subsequent ubiquitination and degradation may underlie the reduction of the p65 nuclear/cytoplasmic ratio induced by CsnB in LPS-stimulated GM-MDMs. This hypothesis was also supported by the increment in the percentage of cytosolic p65 in CsnB-treated GM-MDMs. Although CsnB can also block LPS-induced IκBα degradation in M-MDMs, it did not globally modify the nuclear localization of p65 in these cells. Therefore, it appears that hampering LPS-induced IκBα degradation is a common effect of CsnB in both macrophage subtypes; thus, the different p65 activation outcomes caused by CsnB in GM-MDMs and M-MDMs should involve additional levels of regulation of the NF-κB signaling pathway, such as IκBα resynthesis rate and the consequent p65 nuclear export, p65 synthesis/degradation, and p65 posttranslational modifications (Saccani et al., 2004, Giridharan and Srinivasan, 2018).

These mechanisms must operate with most of the downstream NF-κB target genes regulated by NR4A receptors since no NBRE sites have been identified in the promoters of inflammatory genes negatively regulated by Nur77, such as *TNF*, *IL6*, *IL12A*, *IL12B*, *IL10*, or *CCL2* (McEvoy et al., 2017). An exception is the *IL1B* promoter, which has a +24 kb enhancer containing an NBRE site that recruits Nur77 after LPS stimulation (Freire and Conneely et al., 2018). Nur77 negatively regulates genes that contain NBRE or NurRE sites in their promoters by recruiting co-repressors (Kurakula et al., 2014). In that respect, CsnB analogues induce the recruitment of Nur77 and the co-repressor *N*-CoR to a region containing an NBRE in the promoter of the *BRE* gene, which leads to its transcriptional repression (Liu et al., 2010). For several NF-κB-dependent genes that lack NBRE sites (e.g., *TNF* and *IL6*), the recruitment of Nur77 to the NF-κB regulatory regions has been confirmed (Freire and Conneely, 2018), leaving open the possibility of a direct NF-κB-Nur77 interaction with the subsequent engagement of co-repressors by Nur77.

The absence of cytokine regulation by CsnB in M-MDMs is a puzzle since this agonist is very effective in modulating the transcription of other genes such as *NR4A1* and, more importantly, the anti-inflammatory function of Nur77 was apparent in these cells when its expression was abolished. Metabolic pathways affect cytokine production in pro- and anti-inflammatory macrophages (Chiba et al., 2017), and recent studies support a crucial role of Nur77 in mitochondrial metabolism that impairs macrophage inflammatory responses (Koenis et al., 2018). Nur77-deficient macrophages fail to down-regulate isocitrate dehydrogenase (IDH) expression and thus accumulate high levels of succinate. These macrophages produce more pro-inflammatory cytokines and reactive radical species in a succinate dehydrogenase-dependent manner during inflammation (Koenis et al., 2018). The higher levels of Nur77 expressed by M-MDMs may condition their production of inflammatory mediators by effective down-regulation of IDH

and absence of succinate accumulation. Hence, CsnB would have no drastic effects in cells where the high levels of Nur77 already repress inflammatory responses. Alternatively, M-MDMs may produce endogenous Nur77 agonists that overcome CsnB activity (Vinayavekhin and Saghatelian, 2011, Lakshmi et al., 2019).

In summary, our results uncover the anti-inflammatory properties of Nur77 in primary human macrophages. The high levels of Nur77 expressed by cells resembling homeostatic tissue-resident macrophages (M-MDMs) may constitute a brake to prevent the synthesis of inflammatory mediators even in the absence of exogenous Nur77 agonists. Maintaining low levels of constitutive Nur77 in GM-MDMs could be relevant to sustain their inflammatory phenotype, both at the steady state and upon TLR activation, but at late time points of inflammatory stimulation, Nur77 is eventually induced to counterbalance and limit this response.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imbio.2022.152299.

References

Bahrami, S., Drabløs, F., 2016. Gene regulation in the immediate-early response process. Adv. Biol. Regul. 62, 37–49. https://doi.org/10.1016/j.jbior.2016.05.001.

Bonta, P.I., van Tiel, C.M., Vos, M., Pols, T.W., van Thienen, J.V., Ferreira, V., et al., 2006. Nuclear receptors Nur77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid and inflammatory responses. Arterioscler. Thromb. Vasc. Biol. 26, 2288–2294. https://doi.org/10.1161/01.ATV.0000238346.84458.5d.

Brubaker, S.W., Bonham, K.S., Zanoni, I., Kagan, J.C., 2015. Innate immune pattern recognition: a cell biological perspective. Annu. Rev. Immunol. 33, 257–290. https://doi.org/10.1146/annurev-immunol-032414-112240.

Chen, J., Huang, X.R., Yang, F., Yiu, W.H., Yu, X., Tang, S.C.W., et al., 2022. Single-cell RNA sequencing identified novel Nr4a1+ Ear2+ anti-inflammatory macrophage phenotype under myeloid-TLR4 dependent regulation in anti-glomerular basement membrane (GBM) crescentic glomerulonephritis (cGN). Adv. Sci. 2022, 2200668. https://doi.org/10.1002/advs.202200668.

Chiba, S., Hisamatsu, T., Suzuki, H., Mori, K., Kitazume, M.T., Shimamura, K., et al., 2017. Glycolysis regulates LPS-induced cytokine production in M2 polarized human macrophages. Immunol. Lett. 183, 17–23. https://doi.org/10.1016/j.imlet.2017.01.012.

Ding, R., Sun, X., Yi, B., Liu, W., Kazama, K., Xu, X., et al., 2021. Nur77 attenuates inflammasome activation by inhibiting caspase-1 expression in pulmonary vascular endothelial cells. Am. J. Respir. Cell Mol. Biol. 65 (3), 288–299. https://doi.org/10.1165/rcmb.2020-0524OC.

Fleetwood, A.J., Lawrence, T., Hamilton, J.A., Cook, A.D., 2007. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. J. Immunol. 178, 5245–5252. https://doi.org/10.4049/jimmunol.178.8.5245.

Freire, P.R., Conneely, O.M., 2018. NR4A1 and NR4A3 restrict HSC proliferation via reciprocal regulation of C/EBPa and inflammatory signaling. Blood 131, 1081–1093. https://doi.org/10.1182/blood-2017-07-795757.

Giridharan, S., Srinivasan, M., 2018. Mechanisms of NF-κB p65 and strategies for therapeutic manipulation. J. Inflamm. Res. 11, 407–419. https://doi.org/10.2147/JIR.S140188.

González-Domínguez, É., Domínguez-Soto, Á., Nieto, C., Flores-Sevilla, J.L., Pacheco-Blanco, M., Campos-Peña, V., et al., 2016. Atypical activin A and IL-10 production impairs human CD16+ monocyte differentiation into anti-Inflammatory macrophages. J. Immunol. 196, 1327–1337. https://doi.org/10.4049/jimmunol.1501177.

Gordon, S., Martinez, F.O., 2010. Alternative activation of macrophages: mechanism and functions. Immunity 32, 593–604. https://doi.org/10.1016/j.immuni.2010.05.007.

Gordon, S., Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. Nat. Rev. Immunol. 5, 953–964. https://doi.org/10.1038/nri1733.

Hamers, A.A., van Dam, L., Teixeira Duarte, J.M., Vos, M., Marinković, G., van Tiel, C.M., et al., 2015. Deficiency of nuclear receptor Nur77 aggravates mouse experimental colitis by increased NF-B activity in macrophages. PLoS ONE 10, e0133598.

Hanna, R.N., Shaked, I., Hubbeling, H.G., Punt, J.A., Wu, R., Herrley, E., et al., 2012. NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. Circ. Res. 110, 416–427. https://doi.org/10.1161/CIRCRESAHA.111.253377.

Hu, Y.W., Zhang, P., Yang, J.Y., Huang, J.L., Ma, X., Li, S.F., et al., 2014. Nur77 decreases atherosclerosis progression in ApoE (-/-) mice fed a high-fat/high-cholesterol diet. PLoS ONE 9, e87313.

Ipseiz, N., Uderhardt, S., Scholtysek, C., Steffen, M., Schabbauer, G., Bozec, A., et al., 2014. The nuclear receptor Nr4a1 mediates anti-inflammatory effects of apoptotic cells. J. Immunol. 192, 4852–4858. https://doi.org/10.4049/jimmunol.1303377.

Koenis, D.S., Medzikovic, L., van Loenen, P.B., van Weeghel, M., Huveneers, S., Vos, M., et al., 2018. Nuclear receptor Nur77 limits the macrophage inflammatory response through transcriptional reprogramming of mitochondrial metabolism. Cell Rep. 24, 2127–2140. https://doi.org/10.1016/j.celrep.2018.07.065.

Kurakula, K., Koenis, D.S., van Tiel, C.M., de Vries, C.J., 2014. NR4A nuclear receptors are orphans but not lonesome. Biochim. Biophys. Acta 1843, 2543–2555. https://doi.org/10.1016/j.bbamcr.2014.06.010.

Lakshmi, S.P., Reddy, A.T., Banno, A., Reddy, R.C., 2019. Molecular, chemical, and structural characterization of prostaglandin A2 as a novel agonist for Nur77. Biochem. J. 476, 2757–2767. https://doi.org/10.1042/BCJ20190253.

Lappas, M., 2014. The NR4A receptors Nurr1 and Nur77 are increased in human placenta from women with gestational diabetes. Placenta 35, 866–875. https://doi.org/10.1016/j.placenta.2014.08.089.

Li, L., Liu, Y., Chen, H.Z., Li, F.W., Wu, J.F., Zhang, H.K., et al., 2015a. Impeding the interaction between Nur77 and p38 reduces LPS-induced inflammation. Nat. Chem. Biol. 11, 339–346. https://doi.org/10.1038/nchembio.1788.

Li, X.M., Lu, X.X., Xu, Q., Wang, J.R., Zhang, S., Guo, P.D., et al., 2015b. Nur77 leads to systemic inflammation in elderly mice. J. Inflamm. (Lond) 12, 40. https://doi.org/10.1186/s12950-015-0085-0.

Li, X.M., Zhang, S., He, X.S., Guo, P.D., Lu, X.X., Wang, J.R., et al., 2016. Nur77-mediated TRAF6 signalling protects against LPS-induced sepsis in mice. J. Inflamm. (Lond) 13, 4. https://doi.org/10.1186/s12950-016-0112-9.

Li, X.M., Yang, T.Y., He, X.S., Wang, J.R., Gan, W.J., Zhang, S., et al., 2017. Orphan nuclear receptor Nur77 inhibits poly (I:C)-triggered acute liver inflammation by inducing the ubiquitin-editing enzyme A20. Oncotarget 8, 61025–61035. https://doi.org/10.18632/oncotarget.17731.

Liu, T.Y., Yang, X.Y., Zheng, L.T., Wang, G.H., Zhen, X.C., 2017. Activation of Nur77 in microglia attenuates proinflammatory mediators production and protects dopaminergic neurons from inflammation-induced cell death. J. Neurochem. 140, 589–604. https://doi.org/10.1111/jnc.13907.

Liu, J.J., Zeng, H.N., Zhang, L.R., Zhan, Y.Y., Chen, Y., Wang, Y., et al., 2010. A unique pharmacophore for activation of the nuclear orphan receptor Nur77 *in vivo* and *in vitro*. Cancer Res. 70, 3628–3637. https://doi.org/10.1158/0008-5472.CAN-09-3160.

Locati, M., Mantovani, A., Sica, A., 2013. Macrophage activation and polarization as an adaptive component of innate immunity. Adv. Immunol. 120, 163–184. https://doi.org/10.1016/B978-0-12-417028-5.00006-5.

Maijenburg, M.W., Gilissen, C., Melief, S.M., Kleijer, M., Weijer, K., Ten Brinke, A., et al., 2012. Nuclear receptors Nur77 and Nurr1 modulate mesenchymal stromal cell migration. Stem Cells Dev. 21, 228–238. https://doi.org/10.1089/scd.2011.0076.

Martinez, F.O., Sica, A., Mantovani, A., Locati, M., 2008. Macrophage activation and polarization. Front. Biosci. 13, 453–461. https://doi.org/10.2741/2692.

Martínez-González, J., Badimon, L., 2005. The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. Cardiovasc. Res. 65, 609–618. https://doi.org/10.1016/j.cardiores.2004.10.002.

Maxwell, M.A., Muscat, G.E., 2006. The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. Nucl. Recept. Signal. 4, e002.

McEvoy, C., de Gaetano, M., Giffney, H.E., Bahar, B., Cummins, E.P., Brennan, E.P., et al., 2017. NR4A receptors differentially regulate NF-B signaling in myeloid cells. Front. Immunol. 8, 7. https://doi.org/10.3389/fimmu.2017.00007.

McMorrow, J.P., Crean, D., Gogarty, M., Smyth, A., Connolly, M., Cummins, E., et al., 2013. Tumor necrosis factor modulates thrombospondin-1 expression in human

inflammatory joint disease through altered NR4A2 activity. Am. J. Pathol. 183, 1243–1257. https://doi.org/10.1016/j.ajpath.2013.06.029.

Pei, L., Castrillo, A., Chen, M., Hoffmann, A., Tontonoz, P., 2005. Induction of NR4A orphan nuclear receptor expression in macrophages in response to inflammatory stimuli. J. Biol. Chem. 280, 29256–29262. https://doi.org/10.1074/jbc.M502606200.

Pei, L., Castrillo, A., Tontonoz, P., 2006. Regulation of macrophage inflammatory gene expression by the orphan nuclear receptor Nur77. Mol. Endocrinol. 20, 786–794. https://doi.org/10.1210/me.2005-0331.

Qin, Q., Chen, M., Yi, B., You, X., Yang, P., Sun, J., 2014. Orphan nuclear receptor Nur77 is a novel negative regulator of endothelin-1 expression in vascular endothelial cells. J. Mol. Cell. Cardiol. 77, 20–28. https://doi.org/10.1016/j.yjmcc.2014.09.027.

Rodríguez-Calvo, R., Tajes, M., Vázquez-Carrera, M., 2017. The NR4A subfamily of nuclear receptors: potential new therapeutic targets for the treatment of inflammatory diseases. Expert Opin. Ther. Targets 21, 291–304. https://doi.org/10.1080/14728222.2017.1279146.

Saccani, S., Marazzi, I., Beg, A.A., Natoli, G., 2004. Degradation of promoter-bound p65/RelA is essential for the prompt termination of the Nuclear Factor B response. J. Exp. Med. 200, 107–113. https://doi.org/10.1084/jem.20040196.

Schilling, E., Weiss, R., Grahnert, A., Bitar, M., Sack, U., 2018. Molecular mechanism of LPS-induced TNF- biosynthesis in polarized human macrophages. Mol. Immunol. 93, 206–215. https://doi.org/10.1016/j.molimm.2017.11.026.

Shao, Q., Shen, L., Hu, L.H., Pu, J., Qi, M.Y., Li, W.Q., et al., 2010. Nuclear receptor Nur77 suppresses inflammatory response dependent on COX-2 in macrophages induced by oxLDL. J. Mol. Cell. Cardiol. 49, 304–311. https://doi.org/10.1016/j.yjmcc.2010.03.023.

Sica, A., Mantovani, A., 2012. Macrophage plasticity and polarization: in vivo veritas. J. Clin. Invest. 122, 787–795. https://doi.org/10.1172/JCI59643.

Stocco, C.O., Lau, L.F., Gibori, G., 2002. A calcium/calmodulin-dependent activation of ERK1/2 mediates JunD phosphorylation and induction of Nur77 and 20-hsd genes by prostaglandin F2 in ovarian cells. J. Biol. Chem. 277, 3293–3302. https://doi.org/10.1074/jbc.M110936200.

Vinayavekhin, N., Saghatelian, A., 2011. Discovery of a protein-metabolite interaction between unsaturated fatty acids and the nuclear receptor Nur77 using a metabolomics approach. J. Am. Chem. Soc. 133, 17168–17171. https://doi.org/10.1021/ja208199h.

Vogel, D.Y., Glim, J.E., Stavenuiter, A.W., Breur, M., Heijnen, P., Amor, S., et al., 2014. Human macrophage polarization in vitro: maturation and activation methods compared. Immunobiology 219, 695–703. https://doi.org/10.1016/j.imbio.2014.05.002.

Wu, H., Li, X.M., Wang, J.R., Gan, W.J., Jiang, F.Q., Liu, Y., et al., 2016. NUR77 exerts a protective effect against inflammatory bowel disease by negatively regulating the TRAF6/TLR–IL-1R signaling axis. J. Pathol. 238, 457–469. https://doi.org/10.1002/path.4670.

Xiong, Y., Ran, J., Xu, L., Tong, Z., Adel Abdo, M.S., Ma, C., et al., 2020. Reactivation of NR4A1 restrains chondrocyte inflammation and ameliorates osteoarthritis in rats. Front. Cell Dev. Biol. 8, 158. https://doi.org/10.3389/fcell.2020.00158.

Yan, J., Huang, J., Wu, J., Fan, H., Liu, A., Qiao, L., et al., 2020. Nur77 attenuates inflammatory responses and oxidative stress by inhibiting phosphorylated IκB-α in Parkinson's disease cell model. Aging (Albany NY) 12, 8107–8119. https://doi.org/10.18632/aging.103128.

You, B., Jiang, Y.Y., Chen, S., Yan, G., Sun, J., 2009. The orphan nuclear receptor Nur77 suppresses endothelial cell activation through induction of IkappaBalpha expression. Circ. Res. 104, 742–749. https://doi.org/10.1161/CIRCRESAHA.108.192286.

Zhan, Y., Du, X., Chen, H., Liu, J., Zhao, B., Huang, D., et al., 2008. Cytosporone B is an agonist for nuclear orphan receptor Nur77. Nat. Chem. Biol. 4, 548–556. https://doi.org/10.1038/nchembio.106.

Zhang, Y.J., Song, J.R., Zhao, M.J., 2019. NR4A1 regulates cerebral ischemia-induced brain injury by regulating neuroinflammation through interaction with NF-B/p65. Biochem. Biophys. Res. Commun. 518, 59–65. https://doi.org/10.1016/j.bbrc.2019.08.008.

Zhao, Y., Bruemmer, D., 2010. NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology. Arterioscler. Thromb. Vasc. Biol. 30, 1535–1541. https://doi.org/10.1161/ATVBAHA.109.191163.

Zhao, D., Qin, L., Bourbon, P.M., James, L., Dvorak, H.F., Zeng, H., 2011. Orphan nuclear transcription factor TR3/Nur77 regulates microvessel permeability by targeting endothelial nitric oxide synthase and destabilizing endothelial junctions. Proc. Natl. Acad. Sci. U S A 108, 12066–12071. https://doi.org/10.1073/pnas.1018438108.