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Liver X Receptors Inhibit Macrophage Proliferation through Downregulation of Cyclins D1 and B1 and Cyclin-Dependent Kinases 2 and 4

Mónica Pascual-García,* José M. Carbó,* Theresa León,* Jonathan Matalonga,* Ruud Out,† Theo Van Berkel,† Maria-Rosa Sarrias,‡ Francisco Lozano,§¶ Antonio Celada,§¶ and Annabel F. Valledor*  

Macrophages serve essential functions as regulators of immunity and homeostasis (1). Macrophage activities include the production of cytokines and other signaling molecules that amplify acute inflammatory responses, Ag presentation and modulation of T cell responses, and the involvement in cholesterol homeostasis (2). Regulation of macrophage proliferation, differentiation, and survival is critical to the overall control of the magnitude, duration, and characteristics of immune and homeostatic responses (3). Among the growth factors that influence myeloid differentiation, M-CSF acts specifically on bone marrow precursors committed to the monocytic/macrophagic lineage to promote their proliferation and differentiation (4). In addition, M-CSF prolongs macrophage survival and enhances their functional activities in vitro (5, 6). Effects of M-CSF are mediated through a type III tyrosine kinase receptor, which is encoded by the proto-oncogene Fms. Binding of M-CSF induces dimerization of this receptor and autophosphorylation of its cytoplasmic tyrosine residues (7, 8), which then associate with a large number of proteins containing Src homology 2 domains that consequently activate numerous signaling pathways. Among these pathways, activation of the MEK/ERK cascade is required for macrophage proliferation (9). At the level of transcription, M-CSF signaling induces the expression of genes associated with progression through the cell cycle, including immediate early genes such as Myc and delayed early genes such as d-type cyclins (10). d-type cyclins form complexes with cyclin-dependent kinases (CDK) 4 and 6, and their activity is crucial for the progression into the S phase of the cell cycle (11). The expression of cyclin E is also induced in response to M-CSF, although at later stages during G1. Cyclin E interacts with CDK2 and helps maintain the retino-blastoma protein in a hyperphosphorylated state during late G1- to-S transition.  

Nuclear receptors constitute a family of ligand-dependent transcription factors that regulate diverse aspects of development and homeostasis (12). Liver X receptors (LXRs) represent a subset of the nuclear receptor superfamily that are regulated by oxidized forms of cholesterol (oxysterols) and intermediate products of the cholesterol biosynthetic pathway (13). Two LXR isoforms have
been identified, LXR α (Nr1h3) and β (Nr1h2), encoded by distinct genes. To regulate positively gene expression, LXR agonists form obligate heterodimers with retinoid X receptors and bind to their target genes by recognizing specific LXR response elements (14). At the physiological level, LXR agonists play important roles as integrators of metabolic and inflammatory signaling (reviewed in Ref. 15). Upon activation by oxysterols, LXR agonists activate transcription of genes involved in cholesterol and fatty acid homeostasis, such as the cholesterol transporters ATP-binding cassette A1 (Abca1) and ATP-binding cassette G1 (Abcg1) and the sterol response element binding protein-1c (Srebp-1c). LXR activation also inhibits transcriptional responses to activation of the TLR4 in macrophages by antagonizing the actions of NF-κB (16) through a process involving protein sumoylation (17). Moreover, LXR activation has been shown to enhance macrophage survival during growth factor deprivation and bacterial infection through the induction of an apoptosis inhibitory protein secreted by macrophages (AIM/SP α) (18, 19).

Recent findings have provided evidence that LXR agonists also regulate proliferation in several cellular models (20–22). Macrophage proliferation plays an important role in the pathogenesis of a number of diseases, including atherosclerosis (23). We therefore investigated whether macrophage proliferation is regulated by the LXR pathway. In this report, we show that LXR agonists inhibit primary macrophage proliferation in response to the growth factor M-CSF, both in vitro and in vivo, leading to accumulation of cells in the G0/G1 phase of the cell cycle. The antiproliferative actions of LXR agonists in macrophages were independent of functional expression of p27KIP1 and the cholesterol transporters ABCA1 or ABCG1. LXR activation resulted in decreased expression of several proteins that mediate progression through the G1 phase or entry into the S phase, such as cyclin D1 (CCND1), CDK2, and CDK4, without affecting their mRNA levels. The expression of cyclin B1 (CCNB1), which participates in mitotic progression, was also downregulated. Our findings suggest a role for LXR agonists in limiting macrophage proliferative responses in the context of inflammation.

Materials and Methods

Reagents

Murine rM-CSF was purchased from R&D Systems (Minneapolis, MN). Murine rIFN-γ was obtained from Pierce Biotechnology (Rockford, IL). T901517 (T1317) was purchased from Cayman Europe (Tallinn, Estonia). Murine rIL-3 was obtained from Sigma-Aldrich (St. Louis, MO). Murine rM-CSF was purchased from R&D Systems (Minneapolis, MN). Murine rIFN-γ was obtained from Pierce Biotechnology (Rockford, IL). T901517 (T1317) was purchased from Cayman Europe (Tallinn, Estonia).

Cell culture and animal models

Bone marrow-derived macrophages (BMDMs) were obtained from 7-wk-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) as described previously (24). Bone marrow precursors were differentiated into macrophages in DMEM (PAALaboratories, Pasching, Austria), supplemented with 20% heat-inactivated FCS (PAALaboratories) and 30% L-cell conditioned medium (L-cell cm). Primary microglia were obtained from the brain cortex of neonatal mice as described (25), with some modifications. Briefly, cortical tissues were homogenized and incubated in tissue-culture plates in the presence of DMEM/10% heat-inactivated FCS. Confluent mixed glial cultures were subjected to mechanical detachment, and microglia were selected by adherence to plastic flasks for 3 h. These microglial populations were >95% pure as measured by FACS analysis using FITC-conjugated anti-mouse F4/80 (eBioscience, San Diego, CA). L-cell cm was obtained from L929 cells grown in DMEM supplemented with 10% heat-inactivated FCS. In some experiments, L-cell cm was used as a source of M-CSF. GM-CSF-containing medium was obtained from the fibroblast cell line 155L cultured in DMEM supplemented with 10% heat-inactivated FCS.

AIM was expressed in human embryonic kidney cells (HEK-293 EBNA), as performed previously for human AIM/SP α (26). Briefly, mouse Aim cDNA was obtained by reverse transcription of mouse spleen total RNA and subsequent PCR amplification with specific primers: 5′-GGGCGGCTAGCGGATCTCCAAACAAAGTG-3′ and 3′-GGCGGCGATCTTCACACACATCAAAATGCTG-5′, which incorporate Nhel and BamHI restriction sites, respectively. The PCR products were cloned into the pCEP.Pu vector, a kind gift from Drs. T Sasaki and R. Timpl (Max Planck Institute for Biochemistry, Martinsried, Germany), and transfected into HEK-293 EBNA cells. Aim-transfected cells were selected in DMEM/10% FCS containing 250 μg/ml G418 and 1 μg/ml puromycin (Sigma–Aldrich). AIM expression was confirmed by Western blot analysis with specific Abs. Cell transfectants expressing AIM or untransfected control cells were grown to confluence and exchanged into serum-free medium. The medium was collected twice every 72 h.

LXR-deficient mice (27, 28) in C57BL/6 background were kindly donated by Dr. David Mangeldorf (University of Texas Southwestern Medical Center, Dallas, TX). p27KIP1-deficient mice, originally generated by Dr. Jim Roberts (29), were kindly provided by Dr. Manuel Serrano (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain). ABCG1-null mice were purchased from Deltangen (San Carlos, CA) (30). ABCA1-deficient mice and apolipoprotein E (ApoE)-deficient mice, initially obtained from The Jackson Laboratory (Bar Harbor, ME), were kindly provided by Dr. Joan Carles Escollá-Gil (Institut de Recerca de l’Hospital de la Santa Creu i Sant Pau, Barcelona, Spain).

All of the protocols requiring animal manipulation have been approved by the ethical committee for experimentation with animals at Parc Científic de Barcelona and Universitat de Barcelona.

Proliferation assay

Cell proliferation was measured as previously described (31), with minor modifications. Quiescent macrophages were stimulated with rM-CSF for 24 h. [3H]thymidine (1 μCi/ml; ICN Pharmaceuticals, Costa Mesa, CA) was added 6 h before the end of the experiment. The cells were fixed in 70% methanol, washed in 10% TCA, and lysed in 1% SDS/0.3 M NaOH. Radioactivity was counted by liquid scintillation using a 1400 Tri-Carb Packard counter (GMI, Minneapolis, MN). Each point was performed in triplicate, and the results were expressed as the mean ± SD. To assess microglial proliferation, microglial populations were incubated with DMEM/10% FCS and L-cell cm. [3H]thymidine incorporation was determined as described above.

Alternatively, macrophages were incubated with L-cell cm and LXR agonists for several periods of time (up to 96 h). The number of viable cells was determined by trypan blue exclusion with a hemacytometer. Each point was performed in triplicate, and the results were expressed as the mean ± SD.

Analysis of DNA content by flow cytometry

Cells (106) were fixed in 70% ethanol, washed in PBS, and incubated in PBS supplemented with 0.05% Triton X-100, 5 μg/ml propidium iodide, and 0.5 μg/ml RNase A (Sigma–Aldrich) for 1 h. Propidium iodide staining was measured using a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Fullerton, CA). Nuclei (12,000) were counted for each histogram and cell cycle distributions, as well as the percentage of cells in sub-G0/G1, were analyzed with the multicycle program (Phoenix Flow Systems, San Diego, CA).

In vivo determination of macrophage proliferative responses

A model of in vivo peritonitis was induced in 6–8-wk-old mice (C57BL/6) by i.p. injection of Con A (40 μg/animal in 1 ml PBS) (Sigma–Aldrich). Three days later, in vivo macrophage proliferation was induced by i.p. injection of 5% L-cell cm in 2 ml DMEM. LXR activation was induced by i.p. injection of T1317 (10 mg/kg) both 18 h before and simultaneously to the proliferating agent. Positive and negative control animals were administered two injections of vehicle (DMSO in DMEM). The cells were obtained by peritoneal lavage 24 h after the last i.p. injection. The cells were fixed in 70% ethanol (in PBS) and subjected to propidium iodide staining as described above. FITC-conjugated Abs against the macrophage surface marker F4/80 were used to determine which of the populations detected by forward scatter/side scatter analysis corresponded to macrophages. For each sample, the macrophage population was gated, and cell cycle distributions were determined as described above. Statistical analysis was performed using the Wilcoxon/Mann–Whitney U test.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted using TRIZol (Invitrogen, San Diego, CA) as recommended by the manufacturer. For cDNA synthesis, 1 μg RNA was subjected to reverse transcription using M-MLV Reverse transcriptase.
FIGURE 1. LXR agonists inhibit macrophage proliferation in a dose-dependent manner. A, BMDMs (10⁵ cells/well) were incubated without M-CSF for 24 h. The cells were treated with the LXR agonists T1317 and GW3965 at the indicated concentrations (8 h before the addition of M-CSF). Control cells were treated with vehicle (DMSO). The cells were then incubated with murine rM-CSF (20 ng/ml) during 24 h. B, Cells at different days after the start of differentiation were starved for 18 h in the presence of T1317 or vehicle (DMSO) and then stimulated with L-cell cm (15% v/v) for 24 h. Cells from day 5 of differentiation were preincubated with LXR agonists or vehicle (DMSO) before addition of 5% GM-CSF–containing medium (C) or recombinant murine IL-3 (5 ng/ml) (D). E, Quiescent primary microglia (10⁵ cells/well) were treated with L-cell cm (15% v/v) in the presence or absence of LXR agonists T1317 or GW3965 (1 μM). [3H]thymidine was added during the last 6 h of treatment, and cell proliferation was measured in triplicate. **p < 0.01 versus treatment with L-cell cm. F, Quiescent macrophages (5 × 10⁴ cells/well) were treated with T1317 (1 μM) at different times before (+) or after (+) the stimulation with rM-CSF (10 ng/ml). Positive control cells were incubated with rM-CSF in the presence of vehicle (DMSO). Starved cells were kept in M-CSF–free medium throughout the experiment. In A–F, [3H]thymidine incorporation from triplicates was measured as an indication of cellular proliferation. *p < 0.05, **p < 0.01 versus treatment with the proliferating agent, #p < 0.01 versus starved cells.

G, Quiescent macrophages were treated with T1317 or vehicle for 10 h. The cells were then stimulated with rM-CSF during 24 h. Control cells (Starved) were kept in M-CSF–free medium during the
Similar results were obtained in two (the growth factor M-CSF (Fig. 1)). Real-time monitoring of PCR amplification was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Data were expressed as relative mRNA levels normalized to the expression values of the gene coding for the ribosomal protein L14 or the rRNA 18S.

**Protein extraction and Western blot analysis**

Cells were washed twice in cold PBS and lysed in lysis solution (1% Triton X-100, 10% glycerol, 50 mM HEPES [pH 7.5], 250 mM NaCl, protease inhibitors, and 1 mM sodium orthovanadate). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-ECL; GE Healthcare Europe, Munich, Germany). Membranes were blocked in 5% milk in TBST. To study the phosphorylation of ERK-1/2, we used mAbs anti-diphosphorylated ERK-1/2 (clone MAPK-YT; Sigma-Aldrich) and anti-total ERK-2 Abs from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin protein expression was measured with an anti-cyclin kit (Rockland, Gilbertsville, PA). Abs against CDK2 and CDK4 were purchased from Santa Cruz Biotechnology. To check for protein loading and transfer, we used anti-mouse α-tubulin (Invitrogen) or anti–β-actin (Sigma-Aldrich). The membranes were washed in TBST and incubated for 1 h with peroxidase-conjugated secondary Abs (Jackson ImmunoResearch Europe, Suffolk, U.K. and Sigma-Aldrich). ECL detection was performed (GE Healthcare Europe GmbH), and the membranes were exposed to x-ray films (Kodak, Rochester, NY).

**JNK activity assay**

JNK activity was measured as described (32). Briefly, cells were lysed with nuclear extract protocols and immunoprecipitated with protein A-Sepharose and anti–JNK-1 Ab. After several washes, the reaction was performed with 1 μg GST-C-JUN (1-169; MBL International, Woburn, MA) as JNK substrate; 20 μM ATP, and 1 μCi [32P]-ATP. The samples were subjected to SDS-PAGE electrophoresis, and the gel was dried and exposed to x-ray films.

**Small interfering RNA**

We used small interfering RNA (siRNA) technology to block the induction of the cholesterol transporters Abcg1 and Abca1 (On Target Plus Smart Pool; Dharmacon, Lafayette, CO) or the transcription factor Srebp-1c (validated siMax siRNA NM_011480; i; Eurofins MWG Operon). Control siRNAs were designed against luciferase (5'-CATACCGGAA-TACTTCG-3') (purchased from Dharmacon). siRNAs were transfected into macrophages by electroporation as described (33). Briefly, macrophages (4 × 10^5 cells in 400 μl DMEM) were mixed with 1.5 μM siRNA in 4-mm gap cuvettes (BTX no. 640; Genotronics) and placed on ice for 15 min. Electroporation was carried out at 2300 μF, 300 V, 13 Ω (~27 ms) with an ECM 600 electroporator (BTX). The samples were placed on ice for 20 min before reconstitution in DMEM/20% FCS/30% L-cell cm. Subsequent assays were carried out 24 h after transfection. The effectiveness of the siRNAs used in this study was checked by real-time PCR.

**Results**

BMDMs represent an appropriate cell model for the study of the molecular events that govern primary cell proliferation. In fact, macrophages were central to the identification of β-type cyclins and CDK4, and the first work showing that cell cycle proteins are the targets for antiproliferative agents was performed in macrophages (34). To study the capability of LXR agonists to modulate macrophage proliferation, we preincubated BMDMs with the synthetic LXR agonists T1317 or GW3965 before the treatment with the growth factor M-CSF (Fig. 1A). [3H]Thymidine incorporation during DNA synthesis was measured as an indication of proliferation (31). In a dose-dependent manner, treatment with LXR ligands inhibited [3H]thymidine incorporation in response to M-CSF. The proliferative response of myeloid cells differentiating in vitro in response to M-CSF can be readily detected after 1 d of differentiation in vitro, with maximal levels of DNA synthesis between days 4 and 6 of in vitro differentiation (data not shown). In all stages of differentiation, LXR activation led to inhibition of cellular proliferation (Fig. 1B). Antiproliferative effects were also observed in cells from day 5 of differentiation stimulated with the cytokines GM-CSF or IL-3 in the presence of LXR ligands (Fig. 1C, 1D). Moreover, LXR agonists also inhibited proliferative responses in primary macroglia, specialized macrophages in the CNS (Fig. 1E).

To understand the time requirements needed for LXR agonists to affect macrophage proliferation, we treated macrophages with the agonist T1317 at different times before and after the stimulation with M-CSF (Fig. 1F). Maximal growth arrest was observed when the cells were treated with the LXR agonist before the stimulation with M-CSF. Simultaneous treatment with the LXR ligand and the growth factor resulted in significant inhibition of proliferation. Similar results were obtained when the LXR agonist was added 2 h after the stimulation with M-CSF, whereas progressive loss of the antiproliferative effects were observed when the agonist was supplied at later time points. Cell cycle distribution studies showed that treatment with T1317 resulted in accumulation of cells in the G0/G1 phase, with inhibited transition to the S and G2/M phases of the cell cycle (Fig. 1G).

To check whether LXR agonists exert antiproliferative actions on macrophages in vivo, we used a murine model of peritonitis induced by Con A. Macrophage proliferative responses were induced by i.p. injection of 5% L-cell cm. Peritoneal cells were stained with propidium iodide for flow cytometry analysis of DNA content. Cell cycle analysis was performed for the cellular entire length of the experiment. The DNA content was determined by flow cytometry after propidium iodide staining. Mean values ± SD of the percentage of cells at each phase of the cell cycle were calculated from triplicates. *p < 0.05 versus starved cells, **p < 0.05, ***p < 0.01 versus M-CSF–treated cells. Similar results were obtained in two (B–G) or three (A) independent experiments.
population expressing the macrophage surface marker F4/80. An increase of the percentage of cells within the S phase of the cell cycle was observed as a response to in vivo injection of L-cell cm. Importantly, treatment with T1317 significantly reduced the percentage of cells in S phase (Fig. 2).

We further analyzed the contribution of LXR isoforms to the antiproliferative effects of LXR agonists in macrophages. BMDMs from wild-type (wT), LXRα-deficient, LXRβ-deficient, or LXRα/β-deficient mice were incubated with LXR agonists and then stimulated with M-CSF at different concentrations (Fig. 3A). [3H]thymidine incorporation assays indicated that the inhibitory effects of the LXR agonists were abolished in macrophages deficient for both LXRα and β, but not in single knockout macrophages, which suggests that both isoforms contribute to the growth arrest mediated by LXR agonists in these cells. These effects were confirmed using cell-counting tests (Fig. 3B). We have previously described that immune cell activating factors, such as LPS and IFN-γ, result in macrophage growth arrest (35). In our hands, such activating factors inhibited proliferation in both wT and LXR-deficient macrophages (Fig. 3C), thus indicating that macrophages deficient in LXRα/β have not lost the capability to undergo growth arrest in response to proinflammatory signals.

We next evaluated whether signaling by M-CSF exerted reciprocal negative effects on either expression of LXR isoforms or the induction of LXR target genes (Fig. 4, Supplemental Fig. 1). The expression of Lxrα and β, as well as of their heterodimeric partners Rxrα and β, was indeed upregulated during the process of macrophage differentiation in response to M-CSF (Fig. 4A, Table I). No expression of Rrxγ was detected in these cells (data not shown). In differentiated macrophages, the presence of M-CSF

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** LXRα and β contribute to the antiproliferative effects of LXR agonists in macrophages. A, Quiescent wT, LXRα-deficient (Lxrα−/−), LXRβ-deficient (Lxrβ−/−), and LXRα/β-deficient (Lxrαβ−/−) macrophages (4 × 10⁴ cells/well) were incubated with T1317 or GW3965 (1 μM) during 18 h and then stimulated with different concentrations of rM-CSF (24 h). [3H]Thymidine incorporation was used as a measurement of cell proliferation. Mean values from triplicates are represented. **B**, Cell counts were performed using wT (upper panel) and LXR-deficient (Lxrαβ−/−) (lower panel) macrophages. The cells (10⁵ cells/well) were grown in L-cell cm (30% v/v) during 4 d in the presence of either DMSO (control cells) or LXR agonists T1317, GW3965 (1 μM each), or 24,25-epoxycholesterol (epoxychol; 10 μM). Fresh agonists were added to the cells every day. Total cell counts were determined by trypan blue exclusion at the indicated time intervals. Triplicates from each condition were used to determine the mean ± SD. **p < 0.01 versus treatment with L-cell cm. C, Quiescent wT and LXR-deficient (Lxrαβ−/−) macrophages (4 × 10⁵ cells/well) were incubated with LPS (100 ng/ml), IFN-γ (10 ng/ml), or T1317 (1 μM) during 18 h and then stimulated with rM-CSF (10 ng/ml) for 24 h. Cell proliferation was measured by [3H]thymidine incorporation. *p < 0.05, **p < 0.01 versus M-CSF treatment. The data are representative of two (B) or three (A, C) independent experiments.
did not inhibit basal expression or the induction of LXR target genes, including the cholesterol transporters *Abca1* and *Abcg1*, the transcription factor *Srebp-1c*, *Apoe*, and CH (*Apoc2*), and phospholipid transfer protein (*Pltp*) (Fig. 4B). These results suggest that M-CSF signaling does not reciprocally exert negative interference on LXR activation.

Nuclear receptor activation has been shown to affect the activity of members of the MAPK family (32, 36). Signaling through the MAPKs ERK-1 and -2 is required for macrophage proliferation in response to M-CSF (9, 24). For this reason, we tested whether LXR agonists affect ERK activity during the macrophage response to M-CSF. Treatment with the growth factor led to increased phosphorylation of ERK-1/2 on both the cytoplasm and nucleus; however, we did not observe decreased ERK activity in the presence of LXR ligands (Fig. 5A). No inhibition of JNK activity was detected either at the doses used in these experiments (Fig. 5B), which suggests that the mechanism of action that accounts for LXR-mediated macrophage growth arrest is not related to decreased activity of these early signaling cascades. These observations are in agreement with the capability of T1317 to still lead to significant macrophage growth arrest even when it is supplied 2 h after the treatment with M-CSF (Fig. 1E). We also considered

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**FIGURE 4.** M-CSF does not interfere with LXR activation or the basal expression of LXR target genes. A. Bone marrow cells were differentiated in vitro in macrophage-differentiating media. The cells were obtained sequentially after each day of differentiation. The expression of *Lxr* and *Rxr* isoforms was determined by quantitative real-time PCR. Gene expression values were normalized to the expression levels of 18S. Fold induction levels were calculated using as a reference the levels of expression of each gene at day 0 (immediately after the extraction of the bone marrows). B. Starved differentiated wT macrophages were incubated for 24 h in the presence or absence of rM-CSF (30 ng/ml) and then stimulated with T1317 (1 μM) or vehicle for 18 h. As a negative control of LXR activation, starved LXR α/β-deficient macrophages were incubated with vehicle or T1317. The expression of LXR target genes was evaluated by quantitative real-time PCR and normalized to the expression levels of L14. Fold induction values were calculated using the expression levels of starved wT cells as a reference. The graphics show mean levels ± SEM from two (A) or three (B) independent experiments. *p < 0.05, **p < 0.01 versus cells from day 0 (A) or control cells without agonist (B). n.d., nondetectable levels.
the possibility that regulation of the apoptosis inhibitory factor AIM/SP α could, in an autocrine manner, play a role in mediating the antiproliferative actions described in this study. AIM/SP α is a member of the scavenger receptor cystein-rich superfamily that is upregulated by LXR agonists in macrophages (18, 19). In combination with TGF-β, AIM/SP α mediates growth inhibition of B lymphocytes (37). We overexpressed murine AIM in human embryonic kidney 293 cells and used the supernatants to assess their effect on the proliferation of BMDMs. Control supernatants were obtained from cells transfected with an empty vector. In our hands, no inhibition in the proliferation rate of macrophages was observed (Fig. 5C), although the supernatant containing AIM did increase macrophage survival in response to growth factor withdrawal, as indicated by the analysis of the percentage of sub-G0/G1 nuclei by flow cytometry (Fig. 5D). These observations suggest that autocrine production of AIM is unlikely to account for the LXR-mediated inhibition of macrophage proliferation.

We further characterized changes in the expression of molecules involved in the regulation of the cell cycle. The expression of several cyclins and CDKs was upregulated by M-CSF, as analyzed by Western blotting (Fig. 6A). Interestingly, LXR activation led to decreased protein expression of CCND1 and -B1 and of CDK2 and -4 (Fig. 6A). We also observed inhibitory effects of the LXR agonist on the mRNA expression of CcnB1 at prolonged times of M-CSF treatment, but the mRNA expression of CcnD1, Cdk2, or Cdk4 (Fig. 6B, Supplemental Fig. 1C) was not significantly inhibited, which suggests that downregulation of these molecules is not a general result of direct LXR-mediated transrepression of their genes. Previous work described by other investigators in vascular smooth muscle cells and prostate cancer cells suggested a role for LXR in the regulation of the expression of S phase kinase-associated protein 2 (SKP2) (21, 38), a protein involved in cellular degradation of the cell cycle inhibitor p27KIP1 (39). In our hands, we have not observed changes in the expression of Skp2 mRNA levels in response to LXR activation in macrophages (Fig. 6C, Supplemental Fig. 1D), and LXR agonists exerted antiproliferative effects in p27KIP1-deficient macrophages (Fig. 6D), which suggests that the effects mediated by LXRs on macrophage cyclin/CDK protein levels and proliferation are independent of p27KIP1. Similar results were obtained in macrophages deficient for p21WAF (Fig. 6E).

Recent findings indicated that upregulation of the cholesterol transporter ABCG1 by LXR agonists limit the mitogen-driven expansion of T lymphocytes (20). The authors hypothesized that changes in the intracellular compartmentalization of sterols could probably be sensed by components of the cell-cycle machinery. As a consequence, conditions in which sterols are not readily available in a specific compartment could be interpreted by the cell as a nonappropriate metabolic condition for cell division. We analyzed whether alterations in the expression of key molecules involved in macrophage cholesterol homeostasis affected the capability of LXR agonists to inhibit macrophage proliferation. We used siRNA to downregulate the LXR-mediated induction of Abcg1 (Supplemental Fig. 2A). However, we did not observe changes in the capability of T1317 or GW3965 to inhibit macrophage proliferation in response to M-CSF (Fig. 7A). The use of macrophages derived from ABCG1-deficient mice confirmed these results (Fig. 7B). Interestingly, ABCG1-deficient macrophages showed increased levels of proliferation in response to M-CSF, but activation of the LXR pathway still led to significant inhibition of macrophage proliferation in these cells. We also tested the role of ABCA1 by using either macrophages deficient in Abca1 or ABCG1-deficient cells (Fig. 7C). Interestingly, ABCA1-deficient macrophages showed increased levels of proliferation in response to M-CSF, but activation of the LXR pathway still led to significant inhibition of macrophage proliferation in these cells. We also observed inhibition of macrophage proliferation in response to M-CSF, but activation of the LXR pathway still led to significant inhibition of macrophage proliferation in these cells. We also observed inhibition of macrophage proliferation in response to M-CSF, but activation of the LXR pathway still led to significant inhibition of macrophage proliferation in these cells. We also observed inhibition of macrophage proliferation in response to M-CSF, but activation of the LXR pathway still led to significant inhibition of macrophage proliferation in these cells.
that the LXR pathway affects macrophage cyclin expression and proliferation independently of the changes in cholesterol trafficking caused by the induction of ABCA1 or G1.

Discussion
The combination of anti-inflammatory and prosurvival effects suggests that the LXR pathway may have evolved as a means to potentiate the role of the macrophage in resolution of inflammation. In this work, we have shown that LXR agonists, in a dose-dependent and LXR-specific manner, are capable of limiting the expansion of macrophages and their progenitor cells in response to M-CSF. Recent work demonstrated that activation of the LXR pathway takes place after phagocytosis of apoptotic bodies (40), and apoptotic uptake is known to inhibit the proliferation of macrophages in response to M-CSF (41), which raises the question of whether this effect is mediated through production of LXR ligands.

LXR agonists have been demonstrated to exert antiproliferative actions in other cellular systems as well, including vascular smooth muscle cells (21), prostate (38), and breast (22) cancer cells, T lymphocytes (20), and pancreatic islet β cells (42). While this work was under revision, Kim et al. (43) also demonstrated antiproliferative effects of LXR agonists in several other cell lines, including human THP-1 macrophages. Our results indicate that both LXR α and β isoforms participate in the antiproliferative actions of LXR agonists in macrophages. In the absence of both isoforms, macrophages show an increased proliferative response to M-CSF, and the inhibitory capability of synthetic LXR agonists is abolished. Increased proliferation of glial cells has been also observed in the brains of LXR α/β-deficient mice (44). These observations contrast with the specific requirement of LXR β for the inhibition of mitogen- and Ag-driven expansion of T lymphocytes mediated by oxysterols (20), which may be a consequence of LXR β playing a more predominant role than LXR α in T cells.

The antiproliferative effects of the LXR pathway in BMDMs correlate with inhibitory effects on the protein expression levels of selected cyclins and CDKs. In particular, we have observed reduced protein expression of CCND1 and -B1 and of CDK2 and -4, although we cannot discard that other cyclins and CDKs not tested in this study are also affected. Downregulation of CCND1, CDK2, and CDK4 correlates with the fact that macrophages treated with LXR agonists predominantly accumulate in the G1 phase of the cell cycle. LXR activation only affected protein expression of these molecules without downregulating their mRNA levels, which suggests that, in contrast to the effects of LXR agonists on proinflammatory gene expression, inhibition of CCND1, CDK2, and CDK4 expression is not mediated by direct transrepression of the genes encoding these proteins. Interestingly, expression of CCNB1, which acts later during the cell cycle (45), was affected both at the protein and mRNA levels. We cannot discard, though, that the actions on CCNB1 expression are secondary effects resulting from abnormal progression through the S phase of the cell cycle. LXR activation only affected protein expression of these molecules without downregulating their mRNA levels, which suggests that, in contrast to the effects of LXR agonists on proinflammatory gene expression, inhibition of CCND1, CDK2, and CDK4 expression is not mediated by direct transrepression of the genes encoding these proteins. Interestingly, expression of CCNB1, which acts later during the cell cycle (45), was affected both at the protein and mRNA levels. We cannot discard, though, that the actions on CCNB1 expression are secondary effects resulting from abnormal progression through the S phase of the cell cycle. LXR activation only affected protein expression of these molecules without downregulating their mRNA levels, which suggests that, in contrast to the effects of LXR agonists on proinflammatory gene expression, inhibition of CCND1, CDK2, and CDK4 expression is not mediated by direct transrepression of the genes encoding these proteins. Interestingly, expression of CCNB1, which acts later during the cell cycle (45), was affected both at the protein and mRNA levels. We cannot discard, though, that the actions on CCNB1 expression are secondary effects resulting from abnormal progression through the S phase of the cell cycle. LXR activation only affected protein expression of these molecules without downregulating their mRNA levels, which suggests that, in contrast to the effects of LXR agonists on proinflammatory gene expression, inhibition of CCND1, CDK2, and CDK4 expression is not mediated by direct transrepression of the genes encoding these proteins. Interestingly, expression of CCNB1, which acts later during the cell cycle (45), was affected both at the protein and mRNA levels. We cannot discard, though, that the actions on CCNB1 expression are secondary effects resulting from abnormal progression through the S phase of the cell cycle. LXR activation only affected protein expression of these molecules without downregulating their mRNA levels, which suggests that, in contrast to the effects of LXR agonists on proinflammatory gene expression, inhibition of CCND1, CDK2, and CDK4 expression is not mediated by direct transrepression of the genes encoding these proteins. Interestingly, expression of CCNB1...
showing effects of LXR agonists on p27KIP1 protein levels are based on experiments performed with high doses of these agonists (up to 10 times superior to the maximal doses used in our experimental conditions) (42, 46), and no demonstration is included of whether these effects are LXR specific or not.

Another main point of discussion is whether changes in cholesterol redistribution play an antiproliferative role in macrophages. Bensinger et al. (20) elegantly showed that T cell proliferation correlated with increased expression of the sulfotransferase SULT2B1, an enzyme that transfers sulfate groups to oxysterols, thus inactivating them as LXR ligands and facilitating their export from the cell. As a consequence, expression of the LXR target genes Abca1 and Abcg1 was downregulated during T cell proliferation. Based on the requirement of intracellular
cholesterol for mitosis (48), reduced expression of the cholesterol transporter ABCG1 was proposed to affect the intracellular distribution of cholesterol and represent a strategy for T cells to ensure adequate progression through the cell cycle. Independent groups have indeed reported proliferative advantages in ABCG1 null lymphocytes (20, 49) and in human prostate cancer cells with knockdown expression of ABCA1 (50). In our studies, macrophages with deficient expression of ABCA1 or ABCG1 did show higher proliferation levels compared with control cells, which supports the idea that increased availability of intracellular cholesterol favors proliferative responses. However, M-CSF signaling did not negatively interfere with basal or agonist-induced expression of LXR targets involved in macrophage lipid metabolism, including ABCA1 and ABCG1, which suggests that macrophage

**FIGURE 7.** LXR agonists inhibit macrophage proliferation in the absence of functional ABCG1 or ABCA1 expression. Macrophages from different sources (see below) were treated with rM-CSF (20 ng/ml) in the presence or absence of 1 μM T1317 or GW3965. Control cells were treated with vehicle (DMSO). Proliferation was determined by [3H]thymidine incorporation. A, Macrophages were previously transfected with siRNA against Abcg1 (siAbcg1) or luciferase (siGl3). Similar results were obtained in four independent experiments. B, Macrophages were obtained from wT or ABCG1-deficient mice (Abcg1<sup>−/−</sup>). C, Macrophages were obtained from wT or ABCA1-deficient mice (Abca1<sup>−/−</sup>). D, Macrophages derived from wT or ABCG1<sup>−/−</sup> mice were transfected with siRNA against Abca1 (siAbca1) or luciferase (siGl3). E, Macrophages from wT or Abcg1<sup>−/−</sup> mice were treated with M-CSF in the presence or absence of T1317 and/or probucol (20 μM). F, Macrophages were obtained from wT or Apoe<sup>−/−</sup> mice. G, Macrophages were transfected with siRNA against Srebp-1c (siSrebp-1c) or luciferase (siGl3). In B–G, similar results were obtained in duplicate experiments, each performed in triplicate. In all proliferation tests, *p < 0.05, **p < 0.01 versus the corresponding M-CSF treatment.
proliferation in response to M-CSF, in contrast to mitogen-driven T cell expansion, does not require downregulation of LXR-dependent pathways. In support of this observation, gene profiling studies performed in human macrophages treated with M-CSF revealed enrichment of gene expression programs involved in lipid metabolism (51). Moreover, the antiproliferative effects of LXR agonists on macrophage proliferation were not abolished in macrophages with reduced expression of ABCA1 or ABCG1. The complex nature of macrophage cholesterol homeostasis may help explain these differences between macrophages and T cells. A recent study comparing different cell lines proposed a close correlation between LXR-mediated cell cycle arrest and lipogenic gene expression and triacylglyceride accumulation (43). In our hands, however, the use of siRNA to downregulate Srebp-Ic expression did not counteract the antiproliferative role of LXRs in macrophages, in line with results obtained in human breast cancer cells (22).

Macrophage proliferation has been associated with the pathogenesis of certain diseases. For example, proliferation of macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); in glomerulonephritic disease, macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); in glomerulonephritic disease, macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); in glomerulonephritic disease, macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); in glomerulonephritic disease, macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); in glomerulonephritic disease, macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); in glomerulonephritic disease, macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); in glomerulonephritic disease, macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); in glomerulonephritic disease, macrophage-derived foam cells is critical in the evolution of atherosclerotic lesions (52); 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Supplemental Figure 1. Analysis of the expression levels of 18S or Hprt. A, 18S levels were measured during macrophages differentiation as described in Fig. 4A. The graphic shows mean levels ± SEM from two independent experiments. B-D, macrophages were treated as described in Fig.4B (B), Fig.6B (C) and Fig.6C (D). The expression of hypoxanthine guanine phosphoribosyl transferase (Hprt) was evaluated by real time-PCR and normalized to the expression levels of L14. Each graphic shows mean levels ± SEM from three independent experiments.
Supplemental Figure 2. Blockage of the expression of Abca1, Abcg1 and Srebp-1c by siRNA technology. Macrophages were transfected with siRNAs as described in Fig. 7. mRNA expression levels for Abcg1, Abca1 and Srebp-1c were determined by real time-PCR and normalized to L14 expression levels. The data represent mean values from two independent experiments. *p<0.05; **p<0.01.