Liver X Receptor Activation Stimulates Iron Export in Human Alternative Macrophages

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Rationale: In atherosclerotic plaques, iron preferentially accumulates in macrophages where it can exert pro-oxidant activities.

Objective: The objective of this study was, first, to better characterize the iron distribution and metabolism in macrophage subpopulations in human atherosclerotic plaques and, second, to determine whether iron homeostasis is under the control of nuclear receptors, such as the liver X receptors (LXRs).

Methods and Results: Here we report that iron depots accumulate in human atherosclerotic plaque areas enriched in CD68 and mannose receptor (MR)-positive (CD68+MR+) alternative M2 macrophages. In vitro IL-4 polarization of human monocytes into M2 macrophages also resulted in a gene expression profile and phenotype favoring iron accumulation. However, M2 macrophages on iron exposure acquire a phenotype favoring iron release, through a strong increase in ferroportin expression, illustrated by a more avid oxidation of extracellular low-density lipoprotein by iron-loaded M2 macrophages. In line, in human atherosclerotic plaques, CD68+MR+ macrophages accumulate oxidized lipids, which activate LXRα and LXRβ, resulting in the induction of ABCA1, ABCG1, and apolipoprotein E expression. Moreover, in iron-loaded M2 macrophages, LXR activation induces nuclear factor erythroid 2-like 2 expression, thereby increasing ferroportin expression, which, together with a decrease of hepcidin mRNA levels, promotes iron export.

Conclusions: These data identify a role for M2 macrophages in iron handling, a process regulated by LXR activation. (Circ Res. 2013;113:1196-1205.)

Key Words: atherosclerosis • iron • macrophages • receptors, cytoplasmic and nuclear

Increasing evidences support a role for cellular iron in the development and progression of atherosclerosis. Epidemiological and experimental studies indicate that atherogenesis is associated with alterations in iron storage and handling in the human body. Increased intraplaque iron deposition promotes oxidative stress and protein and lipoprotein oxidation, factors known to affect plaque stability. These data identify a role for M2 macrophages in iron handling, a process regulated by LXR activation.

Iron homeostasis in macrophages is determined by the balance of iron uptake through the transferrin receptor 1 (TfR1) and LDL receptor-related protein 1 (LRP1), involved in uptake of transferrin- and heme-associated iron, respectively, and by the solute carrier family 11 (proton-coupled divalent metal ion transporters) member 2 (DMT1), involved in the uptake of nontransferrin-bound iron and iron release by ferroportin whose expression is controlled by hepcidin and by the transcription factor NR2F2 (nuclear factor erythroid 2-like 2). Within the cells, iron is retained by ferritin (heavy chain and light chain), the latter being most efficient, whereas ceruloplasmin is a ferroxidase facilitating the transfer of macrophage-released iron to transferrin.

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Macrophages are plastic cells that respond to environmental signals (mobilized products, damaged cells, activated lymphocytes, cytokines) by acquiring distinct functional phenotypes. Whereas Th1 cytokines (IFNγ and IL1-β) or bacterial lipopolysaccharide induce a classical proinflammatory profile (M1), Th2 cytokines, such as IL-4 and IL-13, induce an alternative anti-inflammatory and reparatory phenotype (M2). Moreover, in vivo, several macrophage subpopulations have been identified in human atherosclerotic plaques.13,14 We have previously identified CD68+MR+, a population of alternative macrophages coexpressing the pan-macrophage CD68 and the alternative differentiation marker mannose receptor (MR), characterized by a reduced ability to handle lipids but highly competent for phagocytosis.15 Moreover, CD68+MR−CD163+ alternative M2 macrophages have been detected in areas of hemorrhage.16 Such macrophages, induced in vitro by the hemoglobin/haptoglobin complex, produce anti-inflammatory factors and are protected against lipid accumulation.17,18 The objective of this study was, first, to better characterize the iron distribution and metabolism in macrophage subpopulations in human atherosclerotic plaques and, second, to determine whether iron homeostasis is under the control of nuclear receptors, such as the liver X receptors (LXR).

Methods

Immunohistochemical Analysis

Human atherosclerotic plaques were removed from patients eligible for surgical carotid endarterectomy, recruited at the Cardiovascular Surgery Department, Hospital of Lille, France. Informed consent was obtained from all patients. For immunohistochemical analysis, endogenous peroxidase activity was quenched. Endothelial cells were detected by anti-PECAM1/CD31 (Novus Biological), smooth muscle cells by anti-α-smooth muscle actin, and macrophages by anti-CD68 antibodies (Dako), using N-Histofine Simple Stain (Nichirei Biosciences Inc.). PECAM1 was revealed by blue staining (BCIP/NBT; Vector), α-actin by grey precipitate (Vector SG), and CD68 by red staining (Vector Nova Red). Adjacent sections were stained with goat polyclonal anti-human MR (SantaCruz) or mouse monoclonal anti-4-hydroxy-2-nonenal (4-HNE; Abcam) antibody. Sections of atherosclerotic plaques positive for CD68+MR+ or CD68+MR− were submitted to laser capture microdissection as previously described.13 Macrophage-rich areas were captured from 4 adjacent 8-μm sections and pooled for RNA extraction or for lipid extraction by chloroform/methanol (2:1).

Cell Culture

Human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll density gradient centrifugation. Resting macrophages (RMs) were obtained by 6 days of culture in RPMI 1640 medium (Invitrogen, France) supplemented with gentamicin (40 μg/mL), l-glutamine (2 mmol/L; Sigma-Aldrich, France), and 10% pooled human serum (Abcys, France). To yield alternative differentiated macrophages (M2), recombinant human IL-4 (15 ng/mL; Promocell, Germany) was added at the beginning of differentiation and maintained for 6 days. M1 macrophages were obtained by acute treatment of differentiated RM macrophages with lipopolysaccharide (100 ng/mL; 4 hours). Where indicated, the LXR agonists T0901317 (T09; 1 μmol/L) and GW3965 (1 μmol/L) were added for 24 hours in serum-free medium.

Erythrocytes were isolated from autologous blood. The erythrocyte-containing phase was washed and centrifuged 3x (2000 rpm; 5 minutes; 10°C). On the day of use, erythrocytes were incubated for 1 hour at 37°C with oxidation solution (CuSO4 0.4 mmol/L, and ascorbic acid 5 mmol/L in PBS) to render them senescent and put on macrophages at the ratio of 100 erythrocytes per macrophage.

In Vitro Erythropagocytosis Assay

RM and M2 macrophages were incubated for 16 hours with senescent erythrocytes native or labeled with PKH26 fluorescent dye (Sigma) for flow cytometry (FACS) analysis. Noningested erythrocytes were removed by erythrocyte lysis solution (NH4Cl 140 mmol/L, Tris HCl 17 mmol/L in PBS) and macrophages were incubated for 48 hours in medium without serum before RNA extraction. For FACS analysis, noningested erythrocytes were removed, macrophages directly recovered in PBS-EDTA, filtered with an 80 μm filter, fixed in paraformaldehyde 2% in PBS, and analyzed on a FACS Calibur2 instrument.

RNA Extraction and Analysis

Total cellular RNA was extracted using Trizol (Life Technologies, France). RNA extraction from laser capture microdissection–isolated samples was performed using the Picopure RNA extraction kit (MDS Analytical Technologies). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples displaying a RNA Integrity Number ≥26 were used further for RNA analysis. RNA was amplified in 2 rounds using the ExpressArt TRNucleotide mRNA amplification Nano kit (AmpTec GmbH). For quantitative PCR, RNA was reverse-transcribed using random hexamer primers and Superscript reverse transcriptase (Life Technologies), and cDNAs were quantified on a MX3000 apparatus (Stratagene) using specific primers (Online Table I). mRNA levels were normalized to those of cyclophillin.

siRNA-Mediated RNA Interference

siRNA oligonucleotides corresponding to human LXRα, LXRβ, NRF2, ferroportin (Dharmacon), and scrambled control RNA (Ambion) were used. M2 macrophages were transfected using Dharmafect4 reagent (Dharmacon) and then treated for a further 24 hours in the absence or in the presence of T09 (1 μmol/L) or FeCl3 (100 μmol/L).

Measurement of Iron Content by Ferrozine Assay

RM and M2 macrophages were loaded or not with iron (FeCl3, 100 μmol/L; Sigma-Aldrich) in serum-containing medium for 24 hours. Cellular extracts were obtained in NaOH 50 mmol/L and stored for iron quantification. In the iron release experiment, iron-loaded RM and M2 macrophages were incubated in serum-free medium containing T09 (1 μmol/L) or not for different time periods as indicated. In ferroportin siRNA experiments, cells were transfected before iron loading. Iron quantification in cellular extracts and medium was performed using the ferrozine assay.16 Cell culture medium was dried (65°C; 24 hours) and rehydrated by addition of 100 μL of NaOH 50 mmol/L before iron quantification. Iron release was calculated as (iron medium)/(iron medium+iron in cells)×100.

Ferritin Immunostaining

RM and M2 macrophages were treated with FeCl3 (100 μmol/L) for 24 hours, washed with PBS, fixed with paraformaldehyde (4% in PBS) for 15 minutes, and then incubated overnight with an antihuman ferritin antibody (Acris). After washing, cells were incubated for 30 minutes at room temperature with N-Histofine Simple Stain conjugated with antirabbit immunoglobulin G.

Perls Staining

Iron deposition was revealed by Perls Prussian blue staining in the presence or absence of 0.5% 3,3'-diaminobenzidine for 20 minutes.
Flow Cytometry Analysis of Ferroportin
Unloaded or iron-loaded RM and M2 macrophages were incubated with a rabbit polyclonal antiferroportin antibody (ab85370; Abcam) and a phycoerythrin-labeled goat antirabbit secondary antibody (A21428; Molecular Probes). Cells were analyzed using FACScalibur (BD Biosciences) and data processed with FlowJo xV software.

Lipoprotein Preparation and Measurement of LDL Oxidation
LDL (1.030<d<1.063 g/mL) was isolated by sequential ultracentrifugation from plasma of fasted normolipidemic donors. Iron-loaded RM and M2 macrophages were incubated in RPMI medium containing native LDL (1 mg/mL) for 24 hours. LDL was then isolated from the supernatant by ultracentrifugation and conjugated dienes measured by spectrophotometry at 234 nm as previously described. The LDL electrophoretic mobility was determined by migration on Cellogel (Sebia) and visualized by Ponceau Red staining. Copper-oxidized LDL and native LDL (without cell contact) were used as positive and negative controls, respectively.

Protein Extraction and Western Blot Analysis
Proteins were extracted with hypotonic buffer (50 mmol/L Hepes; pH 7.8; 10 mmol/L KCl; 2 mmol/L MgCl₂; 0.1 mmol/L EDTA; 3 mmol/L DTT; 50 mmol/L NaF; 10 mmol/L Na₃PO₄; 1 mmol/L NaVO₄; and protease inhibitors) added with 0.75% NP40. After centrifugation (5 minutes; 11,000 rpm), the supernatant was recovered as cytoplasmic fraction, whereas hypertonnic buffer (50 mmol/L Hepes; pH 7.8; 50 mmol/L KCl; 300 mmol/L NaCl; 10% glycerol; 3 mmol/L DTT; and protease inhibitors) was added to the pellet. After centrifugation (10 minutes; 14,000 rpm), the supernatant was collected as nuclear fraction. Proteins were separated by SDS-PAGE, transferred to Hybond-C Extra membranes (Amersham), and immunoblotted using antibodies against human NRF2, β-actin, or lamin A/C (Santa Cruz Biotechnology). After incubation with a secondary peroxidase-conjugated antibody (Santa Cruz Biotechnology), immunoreactive bands were revealed using a chemiluminescence ECL detection kit (Amersham) and quantified by densitometry using the Quantity One software.

Transient Transfections
COS cells were transfected with the reporter (LXRE)₃-TK-pGL3 and expression (pCMX-empty or pCMX-hl-Xrt) vectors using jetPEI (Polyplus Transfection, France). Subsequently, cells were incubated for an additional 24 hours with nonfluorescent or autofluorescent oxidized lipids extracted from human atherosclerotic lesions. Luciferase and β-galactosidase activities were measured.

Statistical Analysis
Statistical differences between groups were analyzed by ANOVA and Student t test and considered significant when P<0.05.

Results
Iron Depots Colocalize With M2 Macrophages in Human Atherosclerotic Plaques
Within cells of the atherosclerotic plaques, iron preferentially accumulates in macrophages. Given that different macrophage subpopulations are present in human atherosclerotic plaques, we determined whether iron distribution differs between CD68⁺MR⁺ M2 macrophages and CD68⁺MR⁻ macrophages, which resemble in vitro RM. Perls staining followed by intensity signal quantification revealed that iron deposits (stained in blue) colocalize almost exclusively with the CD68⁺MR⁺ macrophages in human atherosclerotic plaques (Figure 1A and 1B). These iron-loaded CD68⁺MR⁺ macrophages are abundant in areas of neovascularization, as indicated by positive PECAM-1/CD31 endothelial cell staining (Figure 1A), likely resulting from the delivery of erythrocytes through intraplaque hemorrhages after microvessel rupture.

M2 Macrophages Are More Efficient Than RM Macrophages in Iron Loading
Because CD68⁺MR⁺, but not CD68⁺MR⁻, macrophages colocalize with iron deposits in vivo, the ability of M2 macrophages to accumulate iron was assessed using an in vitro model. The expression of genes mediating iron uptake, namely TIR1, LRP1, DMT1, was more abundant in M2 macrophages compared with RM macrophages (Figure 2A–2C; Online Figure IA–IC). Ferritin heavy chain expression was higher in RM macrophages, whereas ferritin light chain, more efficient in iron storage, was higher in M2 macrophages (Figure 2D; Table 1).
Iron Loading of M2 Macrophages Induces a Response Promoting Iron Release

Because M2 macrophages display a high iron accumulation capacity, the response of these cells to iron on the expression of genes involved in iron metabolism was analyzed next. Interestingly, iron exposure induced a more pronounced response of M2 compared with RM macrophages as demonstrated by the decrease of Tfr1, Lrp1, and Dmt1, and the increase of ferritin light chain mRNA in M2 compared with RM macrophages (Figure 2A–2D). Hpcidin pronouncedly decreased on iron loading in M2 compared with RM macrophages, whereas ferroportin expression increased (Figure 2E and 2F). Interestingly, time course experiments indicate that the induction of ferroportin expression by iron in M2 macrophages occurs later than the reduction of hpcidin expression (Online Figure II). Expression of Hmox-1 and Nrf2, markers of oxidative stress, also increased pronouncedly in M2 macrophages (Figure 2G and 2H).

Similar gene expression regulations were observed on incubation with senescent erythrocytes (Online Figure III), indicating that M2 macrophages are more efficient in iron handling compared with RM macrophages, independently of the iron source. Experiments performed on iron-loaded, lipopolysaccharide-activated M1 macrophages indicate that their gene expression profile is intermediary between RM and M2 macrophages (Online Figure IV). Finally, iron-containing, CD68-MR⁺ macrophage-enriched areas of human atherosclerotic plaques isolated by laser capture microdissection displayed higher expression of Nrf2, hpcidin, and ferroportin compared with CD68-MR⁻ macrophage-enriched zones (Online Figure V), thus corroborating the in vitro results on iron-loaded M2 macrophages.

Induction of ferroportin protein on iron loading was stronger in M2 compared with RM macrophages, as illustrated by FACS analysis (Figure 3A). In line, iron-loaded M2 macrophages released more iron compared with RM macrophages (Figure 3B). Moreover, although the percentage of iron release is similar between RM and M2 macrophages at short time periods, given the fact that M2 macrophages accumulate more iron compared with RM macrophages, the absolute amount of iron released is significantly higher in M2 than RM macrophages (Online Figure VI). Altogether, these results suggest that M2 macrophages are more sensitive to iron loading and adapt their response to deal with the iron-induced oxidative damages by inducing mechanisms to release iron when excess of iron accumulates.

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**Figure 2.** Iron loading regulates the expression of genes related to iron metabolism mainly in M2 macrophages. Resting macrophage (RM) and M2 macrophages were loaded or not with increasing iron concentrations (FeCl₃ 25, 50, 100 µmol/L). Tfr1 (A), Lrp1 (B), Dmt1 (C), ferritin light chain (FTL) (D), hpcidin (E), ferroportin (F), Hmox-1 (G), and nuclear factor erythroid 2-like 2 (Nrf2) (H) mRNA levels were measured by quantitative PCR and normalized to cyclophilin mRNA and results expressed as means±SD of triplicate determination relative to the levels in RM without iron set at 1. Statistical significant differences are indicated (t test; RM-treated iron vs RM control: §P<0.05; §§P<0.01; §§§P<0.001; M2-treated iron vs M2 control: *P<0.05; **P<0.01; ***P<0.001).

Online Figure ID and IE). Moreover, ferroportin and ceruloplasmin, genes involved in iron release, were less expressed in M2 macrophages (Figure 2F; Online Figure IF and IG), whereas expression of hpcidin, which degrades ferroportin, was higher in M2 macrophages (Figure 2E; Online Figure IH).
Transcriptional Activity

Oxidized Lipids, Which Induce LXR

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LXR transcriptional activity (Figure 1). Macrophages loaded or not with iron were incubated with native nLDL (nLDL; 1 mg/mL). After a 24-h washout period, nLDL was isolated from medium and the conjugated dienes content (C) and electrophoretic mobility (D) measured. Cupper-oxidized LDL (Ox-LDL) was used as positive control. Statistical significant differences are indicated (*P<0.01; RM iron vs RM control: §§P<0.01; M2 iron vs M2 control: §§§P<0.01; M2 iron vs RM iron: #P<0.05).

To determine whether the enhanced iron release by M2 macrophages has functional consequences, the ability of RM and M2 macrophages to oxidize native LDL was determined. LDL incubated with iron-loaded M2 macrophages displayed higher concentrations of conjugated dienes as well as a higher electrophoretic mobility compared with LDL exposed to iron-loaded RM macrophages (Figure 3C and 3D), indicating a stronger degree of oxidation.

Iron Loading Promotes the Formation of Oxidized Lipids, Which Induce LXRα Transcriptional Activity

Interestingly, iron deposits colocalize with oxidized lipids in atherosclerotic plaques. Therefore, we determined whether CD68⁺MR⁺ macrophages accumulate oxidized lipids in vivo. Lipid autofluorescence and 4-HNE staining, both markers of oxidation, colocalized with iron-positive CD68⁺MR⁺ macrophages, being almost absent in CD68⁺MR⁻ macrophages (Figure 4A).

Because iron loading can generate oxysterols, natural ligands for LXR, the ability of autofluorescent-oxidized lipids in atherosclerotic plaques to modulate LXR transcriptional activity was tested. Therefore, LXR response element reporter and human LXRα expression plasmid–transfected COS cells were treated with autofluorescent-oxidized or control nonfluorescent lipids extracted from atherosclerotic plaques. Autofluorescent-oxidized lipids from CD68⁺MR⁺ macrophage–rich areas, but not neutral lipids from CD68⁺MR⁻ macrophage–rich areas of atherosclerotic plaques, increased LXRα transcriptional activity (Figure 4B). In line, iron loading of M2 macrophages in vitro resulted in increased expression of the LXR target genes ABCA1, ABCG1, and apolipoprotein E (Figure 4D–4F). siRNA knockdown experiments demonstrated that this regulation was dependent on LXRα (Figure 4C–4F), but not on LXRβ (Online Figure VII). Altogether, these results show that iron loading activates LXRα transcriptional activity probably by promoting oxysterol formation.

LXRα Activation Increases Iron Export in Iron-Loaded M2 Macrophages

Because iron loading activates LXRα, the effect of LXR activation on macrophage iron metabolism was determined. Among genes involved in iron uptake, storage, and export, the expression of ferroportin and hepcidin was significantly regulated by the LXR agonists T0901317 and GW3965 (Online Figure VIII). siRNA knockdown experiments demonstrated that these effects are mediated by LXRα and not by LXRβ (Figure 5A–5F). Moreover, siRNA experiments show that LXRα but not LXRβ mediates the induction of hepcidin and ferroportin expression by iron loading (Figure 5G and 5H; Online Figure IX).

Next, we tested whether LXR activation stimulates iron release. M2 macrophages were loaded for 24 hours with FeCl3 and treated for a further 24 hours with T0901317. LXR activation enhanced iron release from iron-loaded M2 macrophages (Figure 6A). Interestingly, ferroportin silencing before iron loading, leading to ≈65% reduction of ferroportin mRNA (Figure 6B), affected basal iron release and blocked the induction...
of iron release by LXR activation, indicating that the effects of LXR are mediated by ferroportin induction (Figure 6A).

Because no putative LXR response element sites were found by silico bioinformatic analysis in the human ferroportin promoter, we tested whether the NRF2 pathway is regulated by LXR. Treatment with T0901317 increased NRF2 mRNA and protein levels in the nuclear fraction of M2 macrophages (Online Figure VIII; Figure 7A and 7B). siRNA knockdown experiments demonstrated that the induction of ferroportin mRNA by T0901317 was abolished by NRF2 siRNA, which can be phagocytosed by macrophages, thus leading to an increase in iron content associated with oxidized lipid deposition.21,26 Moreover, erythrocytes can rapidly lyse, thus releasing hemoglobin, which on oxidization releases heme. The latter can be oxidatively cleaved, thus releasing highly reactive free iron.24 Unbound iron can oxidize lipids and induce cell death, thus potentially promoting atherosclerosis progression. By contrast, iron bound to ferritin or transferrin is less reactive.27,28

Previously, we reported the presence of a CD68\(^+\)MR\(^-\) macrophage subpopulation in human atherosclerotic plaques, which closely resembles in vitro IL-4–polarized M2 macrophages, which are distinct from the CD68\(^+\)MR\(^-\) subpopulation by morphology, localization, and function.13,29 Immunohistological analysis showed that CD68\(^+\)MR\(^-\) macrophages colocalize with iron deposits, whereas CD68\(^+\)MR\(^-\) macrophages are poor in iron, suggesting functional differences in terms of iron handling. In vitro studies on IL-4–polarized M2 macrophages showed that M2 macrophages display an expression profile favoring iron uptake and storage and disfavoring iron release, suggesting that M2 macrophages have a high capacity to accumulate iron. Accordingly, ferritin expression and iron content was higher in M2 macrophages compared with RM macrophages after iron loading in line with the histological observations in plaques.

Interestingly, after iron exposure, M2 macrophages completely changed their phenotype and acquired a phenotype similar to fully differentiated M2 macrophages as evidenced by the expression of markers such as CD206, CD163, and F4/80.30 M2 macrophages have been shown to have a strong capacity to accumulate iron and to be highly reactive to oxidized lipids.27,28

**Erythrophagocytosis Induces LXR Target Gene Expression in M2 Macrophages**

Given that erythrocytes are the major source of iron in atherosclerotic plaques,20 the capacity of RM and M2 macrophages to uptake senescent erythrocytes was studied. In line with their stronger ability to accumulate iron, erythrophagocytosis was higher in M2 than RM macrophages (Figure 8A). Moreover, erythrophagocytic uptake increased ABCA1, ABCG1, apolipoprotein E, and ferroportin expression (Figure 8B–8E).

**Discussion**

Neovascularization is commonly found in atherosclerotic plaques and can lead to intraplaque hemorrhage after vessel rupture. Rupture of microvessels releases erythrocytes, which can be phagocytosed by macrophages, thus leading to an increase in iron content associated with oxidized lipid deposition.21,26 Moreover, erythrocytes can rapidly lyse, thus releasing hemoglobin, which on oxidization releases heme. The latter can be oxidatively cleaved, thus releasing highly reactive free iron.24 Unbound iron can oxidize lipids and induce cell death, thus potentially promoting atherosclerosis progression.

**Figure 4. Oxidized lipids from human atherosclerotic plaques and iron loading induce LXRα transcriptional activity.** A, Representative immunostaining for CD68, MR, and iron depots in human carotid atherosclerotic lesions. Oxidized lipids were revealed by autofluorescence or by anti-4-hydroxy-2-nonenal (4-HNE) immunostaining. B, COS cells were transfected with LXR response element reporter and LXRα expression plasmids and treated with autofluorescent-oxidized or nonautofluorescent lipids extracted from CD68\(^+\)MR\(^-\) and CD68\(^+\)MR\(^+\) macrophage–enriched areas of human atherosclerotic plaques, respectively. M2 macrophages were transfected with scrambled or LXRα siRNA, in the presence or absence of iron (FeCl\(_3\) 100 \(\mu\)mol/L; 24 h) and quantitative PCR analysis of LXRα (C), ABCA1 (D), apolipoprotein E (E), and ABCG1 (F) performed. mRNA levels were normalized to cyclophilin mRNA and results expressed as means±SD of triplicate determination relative to the levels in scrambled siRNA-transfected cells set at 1. Statistical significant differences are indicated (t test; scramble vs scramble iron or siLXRα control: \(*P<0.05; **P<0.01; ***P<0.001; \#P<0.001; \#\#P<0.01; \#\#\#P<0.001; control siLXRα vs LXRα iron: \#P<0.05).
Figure 5. LXRα, but not LXRβ, controls the expression of genes involved in iron export. M2 macrophages were transfected with scrambled, LXRα, or LXRβ siRNA, in the presence or absence of T09 (1 μmol/L; A–F), or transfected with LXRα siRNA, in the presence or absence of iron (FeCl3, 100 μmol/L; 24 h; G–H). Quantitative PCR analysis of LXRα (A), LXRβ (D), ferroportin (B, E, G), and hepcidin (C, F, H) mRNA levels were normalized to cyclophilin mRNA and results expressed as means±SD of triplicate determination relative to the levels in scrambled siRNA-transfected cells set at 1. Statistical significant differences are indicated (t test; scramble vs scramble T09 or siLXR control: *P<0.05; **P<0.01; ***P<0.001; scramble T09/iron vs siLXR T09/iron: §P<0.05; §§P<0.01; §§§P<0.001; siLXR vs siLXR T09; #P<0.05; ##P<0.01).

M2 macrophages also phagocytose erythrocytes more avidly compared with RM macrophages.

The iron handling ability of M2 macrophages suggests a modulatory role of these macrophages in atherosclerosis. Notably, by their increased ability to take up iron from the medium or to phagocytize senescent erythrocytes, associated with their dynamic regulatory response enhancing the release of iron, M2 macrophages could play a role in the recycling of potential detrimental iron and to present it under a less active form, such as bound to ferritin or transferrin. Iron exported by ferroportin is normally bound to transferrin and thus exempt of oxidative capacity. Although human macrophages do not synthesize transferrin,12 we speculate that transferrin in atherosclerotic lesions could accept and bind macrophage-excreted iron.13

Figure 6. LXRα activation enhances iron export via ferroportin. A. M2 macrophages were transfected with scrambled or ferroportin siRNA and loaded with iron (FeCl3, 100 μmol/L; 24 h). After medium removal, cells were treated with or without T09 (1 μmol/L; 24 h). Iron release was calculated as described. B. Iron-loaded M2 macrophages were transfected with scrambled or ferroportin siRNA in the absence or presence of T09 (1 μmol/L; 24 h). Ferroportin mRNA levels were measured by quantitative PCR and normalized to cyclophilin mRNA and results expressed as means±SD of triplicate determination relative to the levels in scrambled siRNA-transfected cells set at 1. Statistical significant differences are indicated (t test; scramble control vs scramble T09 or siRNA ferroportin control: **P<0.01; scramble T09 vs siRNA ferroportin T09: §§§P<0.001).

Furthermore, we identify a novel role for LXRα in the regulation of macrophage iron homeostasis. Notably, treatment of alternative macrophages with T0901317 leads to an increased iron release because of the opposite regulation of ferroportin and NRF2, on the one hand, and hepcidin expression, on the other, which occurs in an LXRα-dependent manner. Interestingly, we also found that
genes involved in iron metabolism after iron exposure is under the control of LXRα. Notably, LXRα siRNA decreased the expression of ferroportin and Nrf2 induced by iron loading, indicating that some physiological effects of iron can occur through the activation of LXRα pathways (Online Figure X). In the regulation of iron metabolism, similar as reported for cholesterol efflux,13,35 we did not observe any compensatory effect of LXRα activation, suggesting that LXRα probably plays a minor role in the control of these human macrophage functions. We have previously shown that M2 macrophages express lower levels of LXRα compared with RM macrophages, accompanied by lower cholesterol efflux capacities.13 Here, we report that activation of LXRα pathway regulates iron metabolism, promoting iron export in M2 macrophages. These data clearly show that, despite a lower expression of LXRα in M2 compared with RM macrophages, this nuclear receptor specifically regulates iron export in M2 macrophages, emphasizing the functional differences between both macrophage subtypes and identifying a novel functional role for LXRα specifically in M2 macrophages.

The fact that iron induces the production of LXR agonists, which in turn enhance the release of excess iron, could constitute a protective mechanism in which LXRα plays a role as central regulator. Moreover, LXRα-dependent increased expression of genes involved in cholesterol efflux after iron loading could thus represent a mechanism by which macrophage lipid content is decreased to protect them from detrimental oxidation because of iron accumulation.

Recently, a subpopulation of CD68+MR+CD163+ alternative M2 macrophages has been detected in areas of hemorrhage in human atherosclerotic plaques.15 Such hemoglobin-stimulated macrophages, obtained in vitro on incubation with the hemoglobin/haptoglobin complex, display reduced intracellular iron content through upregulation of ferroportin, which in turn increases expression of ATP-binding cassette transporters and cholesterol efflux, at least partially via LXRα.15 By contrast, we show that increased iron content in IL-4–polarized M2 macrophages enhances the expression of ABCA1/ABCG1 through an LXRα-dependent mechanism. In line, heme-directed monocyte differentiation, giving rise to the so-called Mhem macrophages, is characterized by the induction of the activating transcription factor-1 as well as HMOX-1 and LXRβ, the latter inducing the expression of both LXRα and ABCA1. These macrophages, which are formed in adaptation to intraplaque hemorrhage, are protected from oxidative stress and are less prone to accumulate lipids and to transform into foam cells.6 However, we found that iron loading increases ABCA1 expression as well as iron export mechanisms, through a direct activation of LXRα, independent of LXRβ.

Although the molecular mechanisms appear different, the final lipid phenotype of these macrophage subtypes seems similar. Thus, we cannot exclude that in human atherosclerotic

Figure 7. LXRα activation induces ferroportin expression via nuclear factor erythroid 2-like 2 (NRF2). M2 macrophages were transfected with scrambled, LXRα, or Nrf2 siRNA, treated or not with T09 (1 μmol/L). mRNA levels of Nrf2 (A) and ferroportin (C) were normalized to cyclophilin mRNA and results expressed as means±SD of triplicate determination relative to the levels in scrambled siRNA-transfected cells set at 1. B, M2 macrophages were treated or not with T09 (1 μmol/L; 24 h), and NRF2 protein was measured in the cytoplasmic and nuclear fraction. Statistical significant differences are indicated (t test; scramble control vs scramble T09 or siRNA T09: §§ P<0.01; ** P<0.05; * P<0.01; scramble T09 vs siRNA T09: §§ §§ P<0.01).

Figure 8. M2 macrophages efficiently phagocytose senescent erythrocytes. A, Phagocytosis of senescent PKH26–labeled erythrocytes (RBC) in resting macrophage (RM) and M2 macrophages after 16 h of incubation. Quantitative PCR analysis of ABCA1 (B), ApoE (C), ABCG1 (D), and ferroportin (E) in M2 macrophages in the absence or presence of senescent RBC. mRNA levels were normalized to cyclophilin mRNA and results expressed as means±SD of triplicate determination relative to the levels in RM set at 1. Statistical significant differences are indicated (t test; *P<0.05; ***P<0.001).
plagues these macrophages can exist together in areas of neo-
vascularization/hemorrhage. It is also tempting to speculate
that the presence of these different macrophage subtypes can
change during atherosclerotic plaque progression, with macro-
phages of the phenotype reported here as a first line of defense
against senescent erythrocytes—and iron-caused damages.

Conclusions
We show that M2 macrophages are highly specialized in iron
handling and that iron loading drives the activation of LXRx α
and the transcription of its target genes involved in cholesterol
efflux. For the first time, we demonstrate that macrophage
metabolism is regulated by LXRx activation. Activation of
LXRx α could modulate atherosclerosis, not only by promot-
ing lipid efflux or decreasing inflammation of macrophages,
but also by enhancing their iron recycling capacities through
increasing iron release.

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What Is Known?

- Monocytes differentiate to functionally distinct proinflammatory classical M1 macrophages and anti-inflammatory alternative M2 macrophages.
- CD68+MR+ M2 macrophages are present in human atherosclerotic lesions.
- Macrophages in human atherosclerotic lesions accumulate iron.

What New Information Does This Article Contribute?

- Iron preferentially accumulates in M2 macrophages in human atherosclerotic plaques.
- On iron exposure, M2 macrophages acquire a phenotype favoring iron release, via a strong increase in ferroportin expression.
- In human atherosclerotic plaques, CD68+MR+ macrophages accumulate oxidized lipids, which activate liver X receptors (LXRα and LXRβ) and induce the expression of their target genes.
- LXR activation induces expression of nuclear factor erythroid 2-like 2 (NRF2) and ferroportin and decreases hepcidin mRNA levels, which together promote iron export.

Novelty and Significance

Monocytes infiltrate the intima of large arteries and differentiate into macrophages. Macrophages are functionally heterogeneous cells adapting their phenotype to the cytokine environment. Th1 cytokines promote the M1 phenotype, whereas Th2 cytokines trigger an alternative M2 phenotype. In atherosclerotic plaques, macrophages are the major cells accumulating iron, which can exert pro-oxidant activities. In this study, we have characterized the distribution and metabolism of iron in macrophage subpopulations in human atherosclerotic plaques and determined whether iron homeostasis is under the control of the LXRs. We found that iron is mostly present in CD68+MR+ alternative M2 macrophage–enriched areas. In vitro IL-4–polarized M2 macrophages display a gene expression profile favoring iron accumulation. On iron exposure, M2 macrophages acquire the ability to release iron, via the induction of ferroportin expression. In human atherosclerotic plaques, CD68+MR+ macrophages accumulate oxidized lipids, which activate LXR. Moreover, in iron-loaded M2 macrophages, LXR activation increases ferroportin expression by a NRF2–dependent mechanism, which, together with a decrease of hepcidin mRNA, promotes iron export. Our work identifies a role for M2 macrophages in iron handling, a process regulated by LXR activation.
Liver X Receptor Activation Stimulates Iron Export in Human Alternative Macrophages
Gaël Bories, Sophie Colin, Jonathan Vanhoutte, Bruno Derudas, Corinne Copin, Mélanie Fanchon, Mehdi Daoudi, Loïc Belloy, Stephan Haulon, Christophe Zawadzki, Brigitte Jude, Bart Staels and Giulia Chinetti-Gbaguidi

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### Online table I: Sequences of primers used

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<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
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<tbody>
<tr>
<td>TFR1</td>
<td>5'- CAT ATA CCC GGT TCA GCC TGG-3'</td>
<td>5'- CGA TCA CAG CAA TAG TCC CAT AGC-3'</td>
</tr>
<tr>
<td>LRP1</td>
<td>5'- TGC AGC AAG GCT GAC GGC TC -3'</td>
<td>5'- GAA CCT ACG CCC TCG CAG GC -3'</td>
</tr>
<tr>
<td>DMT1</td>
<td>5'- AGA GCT TAC AGC TTC CCT TTG C -3'</td>
<td>5'- ACC AAG ATT CCT CCT GCA ATC C -3'</td>
</tr>
<tr>
<td>FTL</td>
<td>5'- TTC CGC GAA TTG GCC GAG GAG AAG C -3'</td>
<td>5'- ATG GCA GCT TTC ATG GCG TCT GG -3'</td>
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<tr>
<td>FTH</td>
<td>5'- ACG CCT CCT ACG TTT ACC TGT C -3'</td>
<td>5'- GCA GCT TCA TCA GTT TCT CAG C -3'</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>5'- TGGGTGGACAAGAATGCTAGAC -3'</td>
<td>5'- CCA TCC ATG GTA CAT GGT CAG -3'</td>
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<tr>
<td>Ceruloplasmin</td>
<td>5'- GGA GAA TGG ATG CTC AGC TGT C -3'</td>
<td>5'- GAT TTC CTC AGC GGC AAT GTA G -3'</td>
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<tr>
<td>HMOX-1</td>
<td>5'- GCC CCA GGA TTT GTC AGA GG -3'</td>
<td>5'- CCT CCT CCA GGG CCA CAT AG -3'</td>
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<tr>
<td>NRF2</td>
<td>5'- AAC CAG TGG ATC TGC CAA CTA CTC -3'</td>
<td>5'- CTC AAA CGT AGCAGA AAC CTC -3'</td>
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<td>Hepcidin</td>
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<td>5'- CAC TTT GAT CGA TGA CAG CAG C -3'</td>
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<td>Cyclophilin</td>
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<td>5'-ATG GTG ATC TTC TGG CTC GTC TGG C -3'</td>
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<tr>
<td>LXRα</td>
<td>5'-AGG GCT GCA AGG GAT TCT TCC -3'</td>
<td>5'-TCT GAC ACA CAC TCC TCC C -3'</td>
</tr>
<tr>
<td>LXRβ</td>
<td>5'-CTC AGT CCA GGA GAT CGT GG-3'</td>
<td>5'-CAC TCT GTC TCG TGT TAG G -3'</td>
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<tr>
<td>ABCA1</td>
<td>5'-GAG TGA AGC CTG TCA TCT ACT G -3'</td>
<td>5'-GTG GAG GAC ACA TAG GAC TTT -3'</td>
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<tr>
<td>ABCG1</td>
<td>5'-CAG TCG CTC CTT AGC ACC-3'</td>
<td>5'-TCC ATG CTC GGA CTC TCT G -3'</td>
</tr>
<tr>
<td>ApoE</td>
<td>5'-GAC ACT GTC TGA GCA GGT GCA A -3'</td>
<td>5'-CCT CCA GTT CCG ATT TGT AGG C -3'</td>
</tr>
</tbody>
</table>
Online figure I. M2 macrophages load more iron than RM macrophages. Q-PCR analysis of TfR1 (A), LRP1 (B), DMT1 (C), FTH (D), FTL (E), ferroportin (F), ceruloplasmin (G) and hepcidin (H) in RM and M2 macrophages. mRNA levels were normalized to cyclophilin mRNA and results expressed as mean ± SD of triplicate determination relative to the levels in RM set at 1 (t test; *P<0.05, **P<0.01, ***P<0.001). RM and M2 macrophages were treated with iron (FeCl₃ 100µmol/L) during 24h. Statistical significant differences are indicated (t test; M2 control vs M2 iron **P<0.01, ***P<0.001; RM control vs RM iron §§P<0.01, §§§P<0.001; M2 iron vs RM iron ## P<0.01). Perls staining (I), ferrozine assay (L) and ferritin immunostaining (M) were performed.
Online figure II. Iron loading regulates ferroportin and hepcidin gene expression in M2 macrophages. Q-PCR analysis of ferroportin (A) and hepcidin (B) in M2 macrophages incubated in the absence or presence of iron (FeCl₃ 50 µmol/L) for different time periods. mRNA levels were normalized to cyclophilin mRNA and results expressed as mean ± SD of triplicate determination relative to the levels in control cells set at 1 (t test; *P<0.05, **P<0.01, ***P<0.001).
Online figure III. Erythrophagocytosis regulates the expression of genes related to iron metabolism mainly in M2 macrophages

RM and M2 macrophages were loaded or not with senescent erythrocytes. TfR1 (A), LRP1 (B), DMT1 (C), FTL (D), hepcidin (E), ferroportin (F), HMOX-1 (G) and NRF2 (H) mRNA levels were measured by Q-PCR and normalized to cyclophilin mRNA and results expressed as mean ± SD of triplicate determination relative to the levels in RM without erythrocytes set at 1. Statistical significant differences are indicated (t test; RM control vs RM RBC or M2 control **P<0.01, ***P<0.001; M2 control vs M2 RBC §§P<0.01, §§§P<0.001, RM RBC vs M2 RBC ##P<0.01, ###P<0.001).
Online figure IV. Iron loading regulates the expression of genes related to iron metabolism mainly in M2 macrophages

RM, M1 and M2 macrophages were loaded or not with increasing iron concentrations (FeCl$_3$, 25, 50, 100 µmol/L). TfR1 (A), LRP1 (B), DMT1 (C), FTL (D), hepcidin (E), ferroportin (F) HMOX-1 (G) and NRF2 (H) mRNA levels were measured by Q-PCR and normalized to cyclophilin mRNA and results expressed as mean ± SD of triplicate determination relative to the levels in RM without iron set at 1. Statistical significant differences are indicated (t test; RM, M1, M2 control vs RM, M1, M2 iron; *P<0.05, **P<0.01, ***P<0.001; RM vs M2 §P<0.05, §§P<0.01, §§§P<0.001; RM vs M1 #P<0.05, ##P<0.01, ###P<0.001).
Online figure V. Genes of iron metabolism are high expressed in iron-containing CD68+MR+ alternative macrophages in human atherosclerotic plaques. Q-PCR analysis of NRF2 (A), hepcidin (B) and ferroportin (C) were performed on RNA from LCM-isolated CD68+MR- and CD68+MR+ macrophage-rich areas. mRNA levels were normalized to cyclophilin mRNA and expressed relative to the levels in CD68+MR- macrophage rich-areas set at 1. Each point corresponds to a single atherosclerotic plaque. The median value is shown (t test; *P<0.05).
Online figure VI. Iron-loaded M2 macrophages display higher iron export capacity.
RM and M2 macrophages were iron-loaded (FeCl₃ 100µmol/L, 24h) followed by different wash-out periods (4, 6, 8 and 24h). Medium iron content was measured by ferrozine assay as described and expressed as nmoles of released iron. Statistical significant differences are indicated (t test; M2 vs M2 4h *P<0.05, **P<0.01, ***P<0.001; RM vs RM 4h §§P<0.01; RM vs M2 ### P<0.001 ).
Online figure VII. LXRβ activation is not affected by iron loading in M2 macrophages. M2 macrophages were transfected with scrambled or LXRβ siRNA, in the presence or in the absence of iron (FeCl₃, 100 µmol/L, 24h) and Q-PCR analysis of LXRβ (A), ABCA1 (B), ApoE (C) and ABCG1 (D) performed. mRNA levels were normalized to cyclophilin mRNA and results expressed as mean ± SD of triplicate determination relative to the levels in scrambled siRNA transfected cells set at 1. Statistical significant differences are indicated (t test; scramble vs scramble iron or siLXRβ control **P<0.01, ***P<0.001; siLXRβ control vs siLXRβ iron §§P<0.01; scramble iron vs Si LXRβ iron; ###P<0.001 ).
Online figure VIII. LXR activation regulates gene expression in iron-loaded M2 macrophages. Q-PCR analysis of ferroportin (A,D), NRF2 (B,E) and hepcidin (D,F) mRNA in iron-loaded (FeCl₃ 100 µmol/L, 24h) M2 macrophages treated or not with T090317 (T09, 1µmol/L) or GW3965 (1µmol/L). mRNA levels were normalized to cyclophilin mRNA and results expressed as mean ± SD of triplicate determination relative to the levels in untreated M2 macrophages set at 1. Statistically significant differences are indicated (t test; *P<0.05, **P<0.01).
Online figure IX. Iron regulates gene expression is not an LXRβ-mediated mechanism. Iron-loaded M2 macrophages were transfected with scrambled or LXRβ siRNA. Q-PCR analysis of ferroportin (A) and hepcidin (B) was performed. mRNA levels were normalized to cyclophilin mRNA and results expressed as mean ± SD of triplicate determination relative to the levels in scrambled siRNA transfected cells set at 1.
Online figure X. LXRα regulates iron export in M2 macrophages. Iron-induced expression of the cholesterol transporters ABCA1/ABCG1 is mediated, at least partially, by LXRα. In addition, LXRα activation reduces the expression of hepcidin and increases the expression of NRF2, resulting in an increased ferroportin expression thus leading to an enhanced iron release. These data indicate that some of the physiological effects of iron can occur through the activation of the LXRα pathways.