Research Communication

Oxysterol mixture and, in particular, 27-hydroxycholesterol drive M2 polarization of human macrophages

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Abstract

Macrophages play a crucial role in atherosclerosis progression. Classically activated M1 macrophages have been found in rupture-prone atherosclerotic plaques whereas alternatively activated macrophages, M2, localize in stable plaque. Macrophage accumulation of cholesterol and of its oxidized derivatives (oxysterols) leads to the formation of foam cells, a hallmark of atherosclerotic lesions. In this study, the effects of oxysterols in determining the functional polarization of human macrophages were investigated. Monocytes, purified from peripheral blood mononuclear cells of healthy donors, were differentiated into macrophages (M0) and treated with an oxysterol mixture, cholesterol, or ethanol, every 4 H for a total of 4, 8, and 12 H. The administration of the compounds was repeated in order to maintain the levels of oxysterols constant throughout the treatment. Compared with ethanol treatment, the oxysterol mixture decreased the surface expression of CD36 and CD204 scavenger receptors and reduced the amount of reactive oxygen species whereas it did not affect either cell viability or matrix metalloprotease-9 activity. Moreover, the oxysterol mixture increased the expression of both liver X receptor α and ATP-binding cassette transporter 1. An enhanced secretion of the immunoregulatory cytokine IL-10 accompanied these events. The results supported the hypothesis that the constant levels of oxysterols and, in particular, of 27-hydroxycholesterol stimulate macrophage polarization toward the M2 immunomodulatory functional phenotype, contributing to the stabilization of atherosclerotic plaques. © 2015 The Authors BioFactors published by Wiley Periodicals, Inc. on behalf of International Union of Biochemistry and Molecular Biology, 42(1):80–92, 2016

Keywords: *27-hydroxycholesterol; cholesterol; oxysterols; macrophage polarization; atherosclerosis*

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1. Introduction

Atherosclerosis is a chronic inflammatory disease as well as a disorder of lipid metabolism. The initial event is a response of endothelial cells (EC) to oxidized modified low-density lipoproteins (oxLDLs), which subsequently accumulate in the intima where resident macrophages are present [1]. The activated ECs and macrophages trigger inflammation and, by releasing several chemokines, allow the recruitment of different circulating immune cells including monocytes. Once in the tissue, monocytes differentiate into mature macrophages (M0) and, depending on microenvironmental conditions, acquire distinct functional phenotypes. Classically activated macrophages, termed M1, release pro-inflammatory and immunostimolatory cytokines whereas alternatively activated macrophages (M2) induce myofibroblast activation and release immunoregulatory cytokines.

High levels of oxLDLs and chronic inflammation are crucial events in determining the evolution of the atherosclerotic process [2]. Although both classically and alternatively activated macrophages have been found in atherosclerotic lesions [2], the M1 phenotype is predominant in rupture-prone plaques [3–6], while M2 macrophages are abundant in more stable ones [7].

Moreover, macrophage accumulation of oxidized forms of cholesterol limits inflammation by activating transcription factors such as liver X receptors (LXRs) [8] whose endogenous ligands are cholesterol derivatives [9]. Importantly, binding sites for LXR-retinoic X receptor (RXR) heterodimers have been identified in the promoter of the ATP-binding cassette transporter 1 (*ABCA1*) gene [10]. In this regard, several *in vivo* and *in vitro* studies have shown that LXR-mediated activation of *ABCA1* is a key process that prevents foam cell formation [11,12] by increasing the reverse cholesterol transport [13].

In addition, both enzymatic and non-enzymatic cholesterol oxidation leads to the generation of oxysterols, which are bioactive lipid derivatives that in macrophages play an important role in the cross-talk between lipid metabolism and immune regulation.

However, the exact role played by macrophages in the progression of the atherosclerotic process and the mechanisms, by which the lesion remains stable or develops into a vulnerable plaque, have not yet been fully clarified.

In the present study, we investigated the effects of an oxysterol mixture (OxMix) and of 27-hydroxycholesterol (27-OH), the oxysterol mainly present in the OxMix [14], on the functional polarization of human macrophages differentiated from circulating monocytes of healthy donors.

2. Experimental Procedures

2.1. Cell Cultures and Treatments

Monocyte cells were purified from peripheral blood mononuclear cells (PBMC) of healthy donors (Human Monocyte Cell Isolation Kit II; Miltenyi Biotec). To obtain macrophages (M0), monocytes, purified from PBMC, were cultured (5×10^5/mL) for 7 days in 24 Lumox Multiwell TC-Qualitaet plates (Greiner Bio One GmbH) with 100 ng/mL rM-CSF (PeproTech) [15]. Macrophages were treated with 20 μmol/L OxMix, 5.6 μmol/L 27-OH, cholesterol or ethanol (EtOH) at its final concentration of 0.072%, every 4 H for a total of 4, 8 (4 + 4), and 12 (4 + 4 + 4) H. The composition of the oxysterol mixture, kindly provided by Prof. Giuseppe Poli (Department of Clinical and Biological Sciences, University of Turin, Italy), was 7α-hydroxycholesterol (4%), 7β-hydroxycholesterol (10%), 5α,6α-epoxycholesterol (8%), 5β,6β-epoxycholesterol (22%), cholestan-3β,5α,6β-triol (6%), 7-oxo-cholesterol (21%), 25-hydroxycholesterol (1%), and 27-OH (28%) (Steraloids, Newport, RI) [14].

Blood samples (buffy-coats) were collected from five fasted volunteer donors admitted to the blood transfusion center of IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) S. Martino-IST (Genoa, Italy) after obtaining informed consent. The study was approved by the Ethical Committee of IRCCS S. Martino-IST (34/2012).

2.2. HPLC Extraction of Oxysterols and Cholesterol

Aliquots of the cell medium (175 μL) were mixed with ethanol (1 mL) and extracted twice with hexane (2 mL). The hexane phases were pooled and dried under a nitrogen flux. The separation of oxysterols and cholesterol was performed in HPLC (Waters, Waters Corporation, Milford, MA) equipped with two pumps, Waters 510 and Photodiode array detector Waters 996, on a column Waters Spherisorb 5 μm ODS2 column with an isocratic elution of 7.5% isopropanol in acetonitrile. 7-Oxo-cholesterol peaked at about 16 Min, 27-OH at about 20 Min, and cholesterol at about 75 Min. Quantification of the cholesterol, 7-oxo-cholesterol and 27-OH was performed by comparison with the elution of standard molecules. The described procedure was modified from that described by Kock [16].

2.3. Fluorescence Microscopy Analysis of Apoptotic and Necrotic Cells

For the assessment of apoptosis and necrosis, cells were analyzed as previously described [17]. Following treatment, cells were incubated with fluorescein isothiocyanate (FITC)-labeled recombinant Annexin-V and propidium iodide (PI; BioVision, Mountain View, CA). Cells were then visualized and counted (four fields of 200–400 cells) by fluorescence microscopy using a Leica DMIRB microscope (Leica, Wetzlar, Germany) with a dual filter set for FITC and rhodamine. Images were acquired with a Leica DCF320 camera. Cell death was evaluated as a percentage of Annexin-V (apoptotic) or PI-positive (necrotic) cells.

2.4. MTT Assay

Cell viability was determined using the dimethylthiazolyl-2-5-diphenyltetrazolium bromide staining (MTT; Sigma). Briefly, cells were seeded in 96-well plates (Corning Incorporated, Corning, NY) and then treated. Next, the cells were incubated with 1.2 mmol/L MTT for 3 H at 37°C. After incubation, the medium was discarded, insoluble formazan precipitates were dissolved in HCl (0.1 mol/L in isopropanol) and the absorbance

at 570 nm was recorded using a microplate reader (EL-808; BioTek Instruments Inc., Winooski, VT).

2.5. Detection of Reactive Oxygen Species (ROS) Production

Detection of ROS was performed as previously described [18]. Briefly, after treatments, the cells were rinsed with 10 mM PBS and incubated with 5 μmol/L dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma) for 30 Min at 37 °C in the dark. At the end, the cells were washed with PBS and incubated with 90% dimethyl sulfoxide for 10 Min in the dark with shaking. Fluorescence at 485 nm ex/520 nm em was measured by a fluorimetric plate reader (FLUOstar Optima; BMG Labtech GmbH, Offenburg, Germany). Values were normalized to the protein content and expressed as arbitrary units (A.U.).

2.6. Flow Cytofluorimetric Analysis

To determine the rate of apoptosis, cells were stained with FITC-conjugated Annexin V (BD Biosciences) according to the manufacturer's instructions. For cytofluorimetric analysis (FACSCalibur; Becton Dickinson, New Jersey), cells were stained with phycoerythrin (PE) or FITC-conjugated monoclonal antibodies (mAbs) or with unconjugated mAbs followed by PE-conjugated isotype-specific goat anti-mouse secondary antibody (Southern Biotechnology, Alabama). In order to avoid non-specific binding to the Fc receptors expressed at the cell surface of macrophages, these cells were pre-incubated for 30 Min at 4 °C with human IgG (Jackson ImmunoResearch Laboratories, Suffolk, UK) before specific mAb staining. The antibodies used were the following: anti-CD80-PE, anti-CD206-FITC, and anti-CD36-FITC (BD Bioscience); anti-CD204-PE, anti-CD74, anti-CXCR4, and anti-CXCR2 (R&D System, MN); anti-IL 18 (MBL, international Corporation, MA), and also anti-CXCR1 (Santa Cruz Biotechnology, Inc, Texas).

2.7. Cytokine Release

Cytokine release was evaluated by the ELISA method. The ELISA kits used were the following: Migration inhibitory factor (Human MIF, Abcam), Interleukin-6 (IL-6, BioSource International, Inc., Belgium), Interleukin-18 (IL-18, Medical Biological Laboratories, Naka-ku Nagoya, Japan), Tumor Necrosis Factor-α (Human TNF-α, UltraSensitive, Invitrogen, Paisley, UK), Interleukin-1β (IL-1β, Human IL-1β, UltraSensitive, Invitrogen), Interleukin-8 (Human IL-8, UltraSensitive, Invitrogen), and Interleukin-10 (Human IL-10, UltraSensitive, Invitrogen).

2.8. Matrix Metalloproteinase (MMP) Activity

MMP activity in the conditioned media was determined by zymography. Cells were cultured and treated in serum-free medium. Subsequently, the conditioned medium was harvested and centrifuged at 13,000 g for 10 Min at room temperature and the resulting supernatant was concentrated with Amicon Ultra Centrifugal Filters (Millipore Ireland Ltd, Country Cork, UK). Total protein amounts were determined by the bicinchoninic acid (BCA) method (Pierce, Thermo Scientific, Rockford, IL) and substrate polyacrylamide gel electrophoresis was carried out as described by Heussen [19] using the gelatine Ready Gel Zymogram 10% (Bio-Rad Laboratories). Briefly, samples were mixed with Laemmli buffer, warmed at 37 °C for 30 Min and subjected to gel electrophoresis. Subsequently, the gel was incubated for 48 H in developing buffer (50 mmol/L Tris pH 7.4, 0.2 mol/L NaCl, 1% Triton X-100, 3 mmol/L sodium azide, and 5 mmol/L $CaCl_2$). The gel was then stained for 1 H (0.2% Coomassie Blue, 30% ethanol, and 10% acetic acid) and de-stained in a solution containing 10% acetic acid [20]. Gelatine digestion was analyzed with an image densitometer connected to the Quantity One software (Bio-Rad Laboratories).

2.9. Immunoblot Analysis

Immunoblots were carried out according to standard methods [21] using anti-β-actin (Sigma), anti-ABCA1 (Abcam, Cambridge, UK), and anti-LXR mAbs (Santa Cruz). The anti-mouse secondary Ab was coupled with horseradish peroxidase (GE Healthcare, Buckinghamshire, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's (GE Healthcare) instructions. Chemiluminescence was monitored by exposure to film and the signals were analyzed under non-saturating conditions with an image densitometer (Bio-Rad Laboratories, Hercules, CA).

2.10. Real-Time (RT)-PCR

cDNA was synthesized by reverse transcription from 2 μg RNA with a commercial kit (High-Capacity cDNA reverse transcription kit; Life Technologies, Monza, Italy) according to the manufacturer's instructions. Singleplex RT-PCR was performed on 40 ng of cDNA using TaqMan gene expression assay kits prepared for human LXRα, LXRβ, β-actin, and TaqMan Fast Universal PCR master mix. The cycling parameters were as follows: 20 Sec at 95 °C (AmpErase UNG activation), 3 Sec at 95°C (AmpliTaq Gold DNA polymerase activation), 40 cycles of 3 Sec at 95 °C (melting), and 30 Sec at 60 °C (annealing/extension). The fractional cycle number at which fluorescence passes the threshold in the amplification plot of fluorescence signal versus cycle number was determined for each tested gene. Results were normalized to the expression of actin and quantified according to the method proposed by Livak and Schmittgen [22].

2.11. Statistical Analyses

Results are expressed as mean ± SD from at least five independent experiments. The statistical significance of parametric differences among the sets of experimental data was evaluated by one-way ANOVA and Dunnett's test for multiple comparisons.

The multivariate analysis, shown in supplementary data (Fig. 1 Supporting Information), was performed by an R-based software developed by the Chemometric Group of the Division of Analytical Chemistry of the Italian Chemical Society, freely downloadable from http://gruppochemiometria.it/gruppo-lavoro-r-in-chemiometria.html.

Principal Component Analysis (PCA) is a multivariate data analysis method which allows the extraction and visualization of the information contained in a multivariate data set. PCA produces a set of new orthogonal variables (the Principal

Components, PC) that are linear combinations of the original variables. The first PC corresponds to the direction explaining the maximum variance (*i.e.,* the maximum information); the second PC corresponds to the direction, orthogonal to the first PC, explaining the maximum variance not explained by the first PC, and so on. By plotting the scores (*i.e.,* the coordinates) of the samples in this new space, an efficient visual representation of the samples can be easily obtained (the closer two samples are, the more similar they are, the farther, the more dissimilar). On the other hand, the loadings (*i.e.,* the coefficients of the linear combinations describing the PC's) indicate the relative contribution of the variables to each PC. As the score plot gives information about the relationships among the samples, in the same way, the loading plot gives information about the relationships among the variables (*i.e.,* variables that are very close are directly correlated, variables that are opposite are inversely correlated and variables that are orthogonal are uncorrelated).

3. Results

3.1. OxMix Reduces the Levels of CD36 and CD204, the Scavenger Receptors Involved in Oxysterol Trafficking

Macrophages (M0) were exposed for 4, 8, or 12 H to OxMix (20 μmol/L) [14], cholesterol (20 μmol/L), or ethanol (0.072%) in the same volume used to dissolve either the oxysterol mixture or cholesterol. The compounds used were supplied to the cells every 4 H without interfering with cell viability, as evaluated by MTT analysis (Fig. 1 Supporting Information).

The HPLC analysis showed that the repeated administration of the compounds maintained the levels in the culture media of 7-oxo-cholesterol (4 μmol/L $\pm$ 0.5) and 27-OH (5.6 μmol/L $\pm$ 0.6), these being the most represented oxysterols in the mixture, and of cholesterol, all constant throughout the treatment (Fig. 1A). At the end of the incubation period, we analyzed the expression of CD36 and CD204, the scavenger receptors involved in oxysterol trafficking [23], by flow cytometric analysis (Fig. 1B, upper panels). As shown in Fig. 1B, after 8 and 12 H of exposure to the OxMix, the levels of CD36 expression at the cellular surface had been reduced, in respect to ethanol, by 20% and 25%, respectively. Moreover, 8 H of OxMix exposure induced a 15% decrease in CD204 levels that did not change after 12 H of treatment (Fig. 1B). Conversely, cholesterol exposure did not significantly alter CD36 or CD204 expression at any time (Fig. 1B).

3.2. OxMix does not Induce Apoptosis and Reduces ROS Levels

As shown in Fig. 2, both fluorescence microscopy (A) and cytofluorimetric Annexin-V analyses (B), demonstrated that neither OxMix, cholesterol, nor ethanol induced macrophage apoptosis. Moreover, the different treatments did not increase ROS generation at either 4 or 8 H but, at 12 H, OxMix reduced ROS levels by 43% in respect to ethanol (Fig. 2C).

Consistent with the observed ROS down-regulation, OxMix and cholesterol did not influence the secretion of MMP-9 (Fig. 2D), the metalloproteinase found to be stimulated by oxidative stress in atherosclerotic lesions [24,25].

3.3. OxMix Stimulates the Secretion of IL-10 and Reduces the MIF Release

Taking into consideration that oxysterols have been reported to induce the expression of various key inflammatory molecules [26], the release of proinflammatory/immunostimulatory factors was investigated. Interestingly, IL-10 secretion increased by 40% and 57% after 8 and 12 H of OxMix treatment, respectively (Fig. 3A). In addition, the release of MIF was reduced by 66% after 12 H of treatment with OxMix (Fig. 3B). Cholesterol alone was able to enhance IL-10 release by 100% after 12 H (Fig. 3A). Furthermore, secretion of IL-1β (Fig. 3C), TNF-α (Fig. 3D), IL-8 (Fig. 3E), and IL-18 (Fig. 3F) was not significantly altered either by OxMix or cholesterol. Under all experimental conditions, the amounts of secreted IL-6 were below the detection limit (data not shown).

3.4. OxMix Increases CXCR2 and CXCR4 Expression

In order to have a global understanding of the data obtained, a PCA was performed. The data set (Fig. 2 Supporting Information) was made up of 20 samples (for each of the 5 subjects, 2 different compounds, and 2 different times) and 16 variables (mIL-18G, mIL-18p, CD80G, CD80p, CD206G, CD206p, CXCR4G, CXCR4p, CXCR2G, CXCR2p, MIF, TNF-α, IL-1β, IL-8, IL-10, and IL-18). PCA analysis demonstrated that the percentages of CXCR2 and CXCR4 positive cells were greater in samples treated with OxMix (Figs. 2 and 3 Supporting Information). These results were confirmed by cytofluorimetric analysis (Fig. 4) that showed that 12 H of treatment with OxMix induced an 80% and 56% increase of CXCR2 and CXCR4 expression, respectively.

3.5. OxMix Increases the Expression of LXRα Receptor and ABCA1

Since cholesterol and its derivatives are able to activate LXR [8], the expression of this transcription factor and of ABCA1, a cholesterol efflux regulatory protein induced by LXR/RXR heterodimers, was analyzed.

LXRα mRNA levels were increased in respect to ethanol, 3-fold after 12 H of treatment with cholesterol and 2.5- and 6-fold after 8 and 12 H of treatment with OxMix, respectively (Fig. 5A, left panel). A similar effect of OxMix was also observed analyzing LXRα protein levels (Fig. 5A, right panel). In particular, 12 H of treatment with cholesterol increased LXRα by 50% in respect to EtOH treatment, whereas OxMix, at 8 and 12 H, further increased LXRα levels by 130% and 270%, respectively. No change was seen in the expression of LXRβ under any of the treatment conditions (data not shown). In addition, the ABCA1 level was enhanced by 70% in respect to ethanol after 12 H of treatment with cholesterol, and by 80% and 260% fold, after 8 and 12 H of exposure to OxMix, respectively (Fig. 5B).

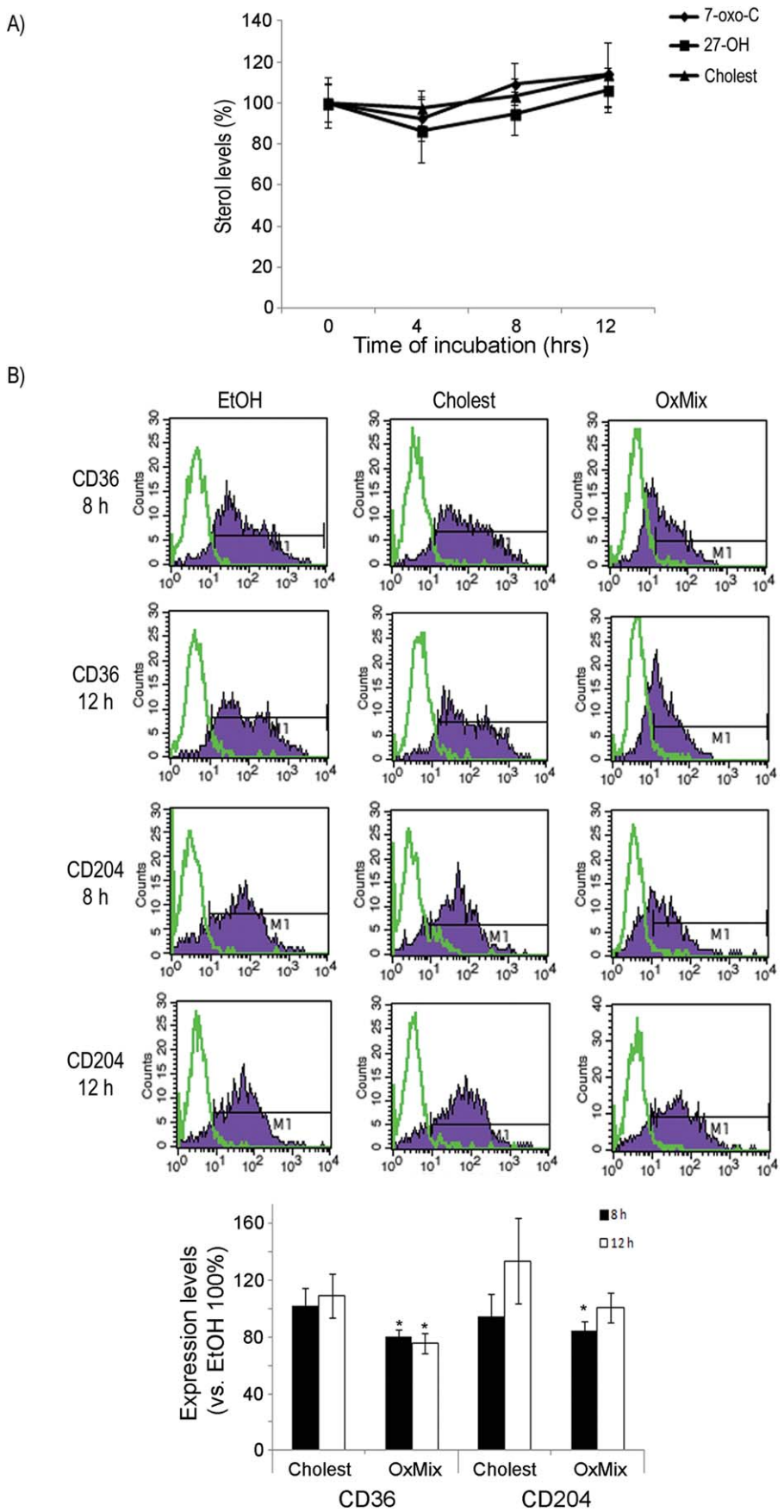

FIG 1

*OxMix treatment reduces the expression of CD36 and CD204 on the membrane of human macrophages. **(A)** Macrophages (M0) were exposed every 4 H, for a total of 4, 8, and 12 H, to ethanol (0.072%, EtOH), cholesterol (Cholest), or oxysterol mixture (OxMix). The levels of 7-oxo-cholesterol (7-oxo-C), 27-OH cholesterol (27-OH), and cholesterol in the medium were determined by HPLC analysis and expressed as percentage variation, in respect to OxMix treatment at time 0. **(B)** Surface expression of CD36 and CD204 were determined by flow cytofluorimetric analysis (upper panels). Plots shown are representative of five independent experiments carried out on cells isolated from five different donors. Graphed data (lower panel) show mean ± standard deviation of total data. *$P < 0.05$ versus EtOH-treated cells.*

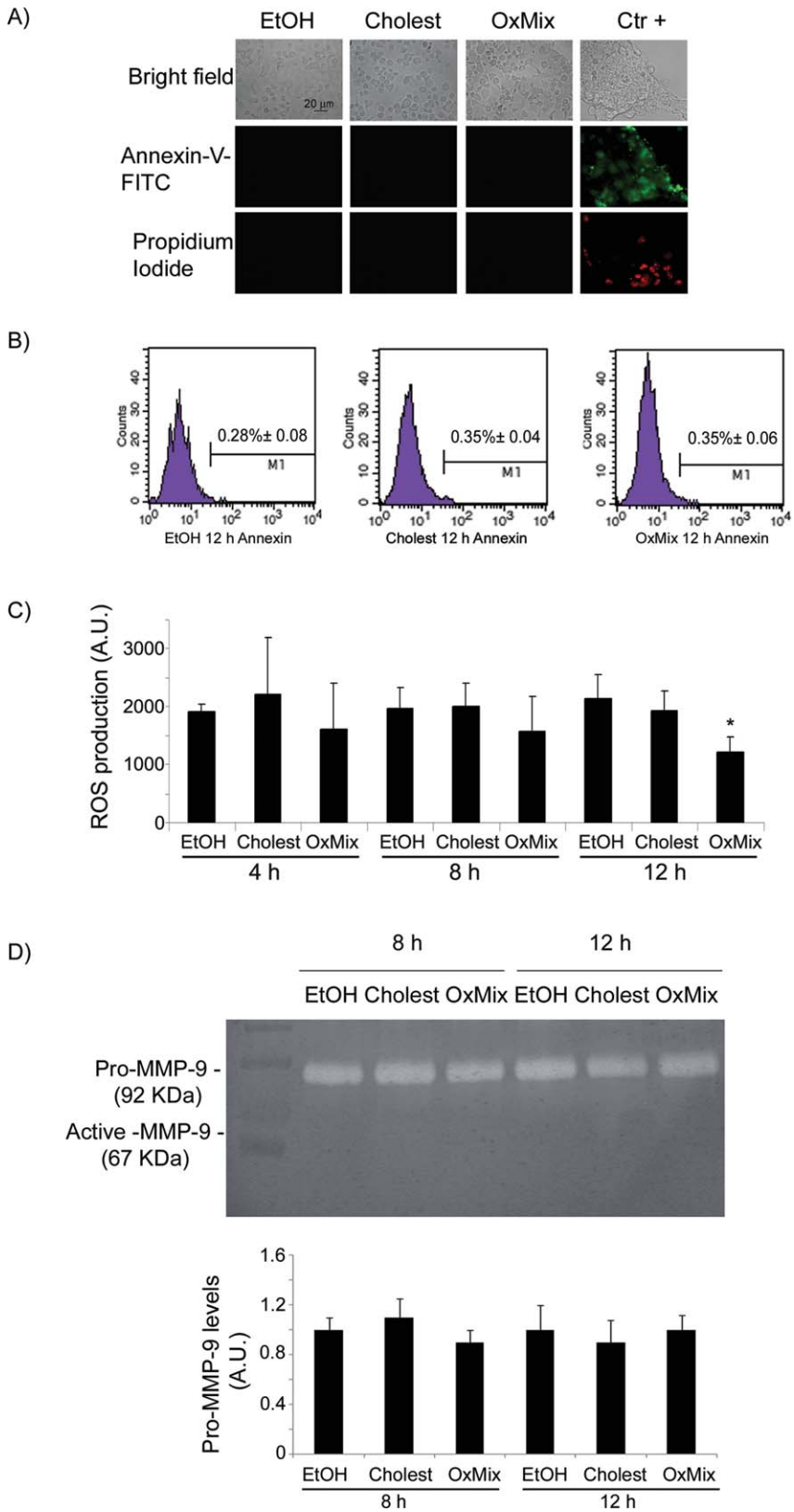

FIG 2

OxMix does not induce macrophage apoptosis, reduces intracellular ROS levels and does not influence MMP activity. ***(A)*** *Representative images of macrophages (M0) exposed for 12 (4 + 4 + 4) H to ethanol (EtOH), cholesterol (Cholest), or oxysterol mixture (OxMix) and labeled with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. Apoptotic cells show green fluorescence (Ctr+) as a result of the binding of AnnexinV to phosphatidylserine, as described in "Materials and Methods" section. Necrotic cells show red fluorescence (Ctr+) as a result of the ability of propidium iodide to penetrate cells that have lost their membrane integrity.* ***(B)*** *Flow cytometry results from annexin-V-FITC stained macrophages (M0) exposed for 12 (4 + 4 + 4) H to ethanol (EtOH), cholesterol (Cholest), or oxysterol mixture (OxMix). Apoptosis rate is indicated in the plots. Data are normalized to FITC-fluorescence signals.* ***(C)*** *Macrophages (M0) were exposed every 4 H, for a total of 4, 8, and 12 H to EtOH, Cholest, or OxMix. ROS analysis was performed as described in "Materials and Methods" section. Graphed data show mean ± standard deviation of the mean for five independent experiments carried out on cells isolated from five different donors. *P < 0.05 versus EtOH-treated cells.* ***(D)*** *MMP9 activity was determined in the conditioned media obtained from macrophages (M0) exposed to EtOH, Cholest, or OxMix for the indicated times. The image of gelatin zymography is representative of five independent experiments with essentially identical results. The histograms summarize quantitative data of means ± SD of five independent experiments carried out on cells isolated from five different donors.*

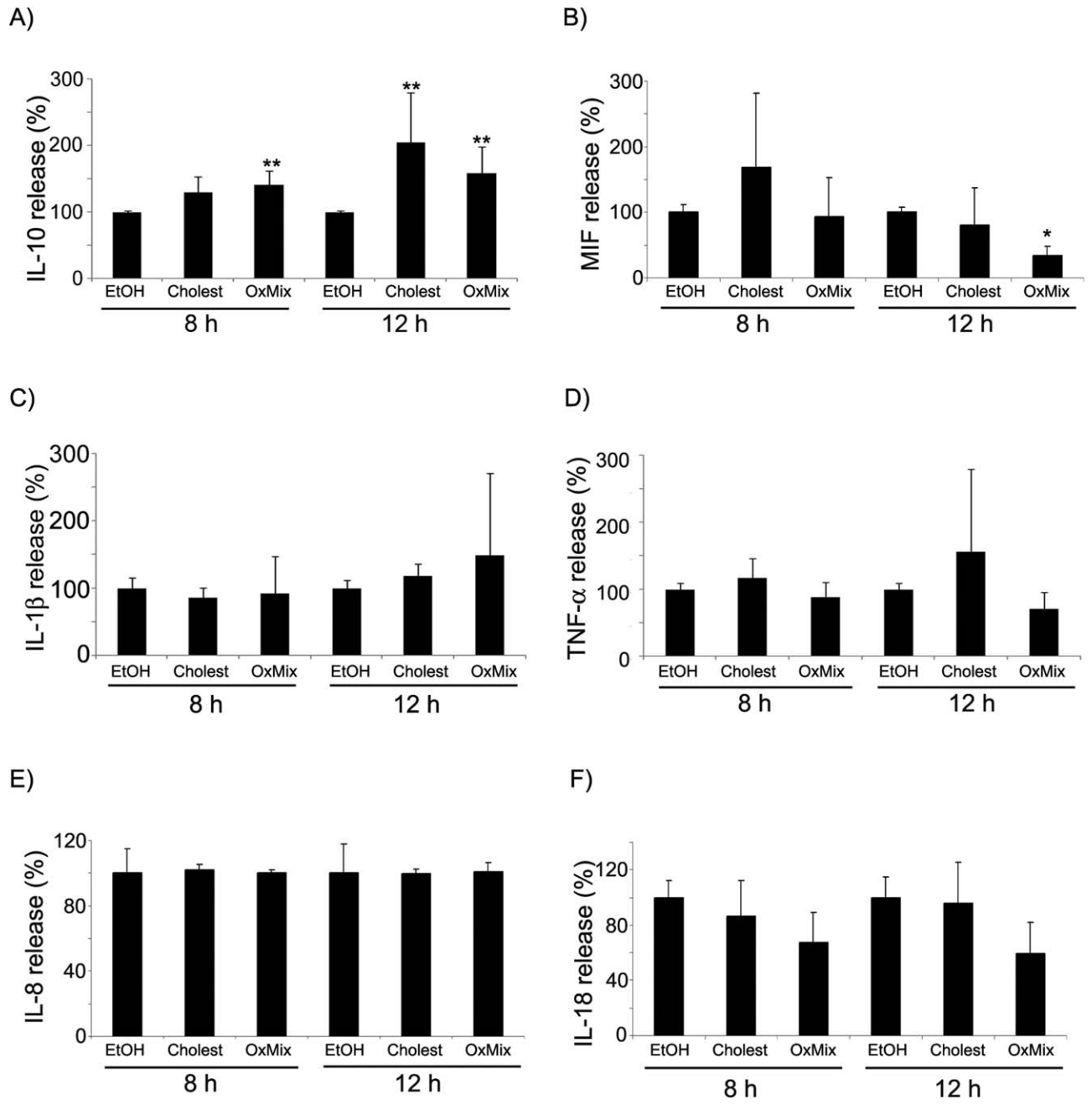

FIG 3

*OxMix stimulates the macrophage secretion of IL-10 and reduces the release of macrophage migration inhibitory factor (MIF). The secretion of IL-10 (**A**), MIF (**B**), IL-1β (**C**), TNF-α (**D**), IL-8 (**E**), and IL-18 (**F**) was evaluated by ELISA as described in "Materials and Methods" section. Macrophages (M0) were exposed for the indicated time to ethanol (EtOH), cholesterol (Cholest), or oxysterol mixture (OxMix). Graphed data show mean ± standard deviation from five independent experiments carried out on cells isolated from five different donors. *$P < 0.05$ versus EtOH-treated cells; **$P < 0.01$ versus EtOH-treated cells.*

3.6. 27-OH Treatment of Macrophages Reduces the Membrane Levels of CD36 and CD204, Stimulates the Secretion of IL-10 and Increases LXR and ABCA1 Expression

Since the OxMix contains about 28% of 27-OH, the major LXR ligand, macrophages (M0) were exposed for 8 or 12 H to 5.6 μM 27-OH, the same amount as present in the OxMix. The oxysterol, supplied to M0 cells every 4 H did not alter cell viability, as evaluated by MTT analysis (Fig. 1 Supporting Information).

Similarly to what was observed with OxMix (Fig. 1B), compared with ethanol, 27-OH treatment reduced the expression of CD36 by 23% and 21% after 8 and 12 H, respectively (Fig. 6A). Moreover, CD204 levels were decreased by 22% only after 12 H of 27-OH exposure (Fig. 6A).

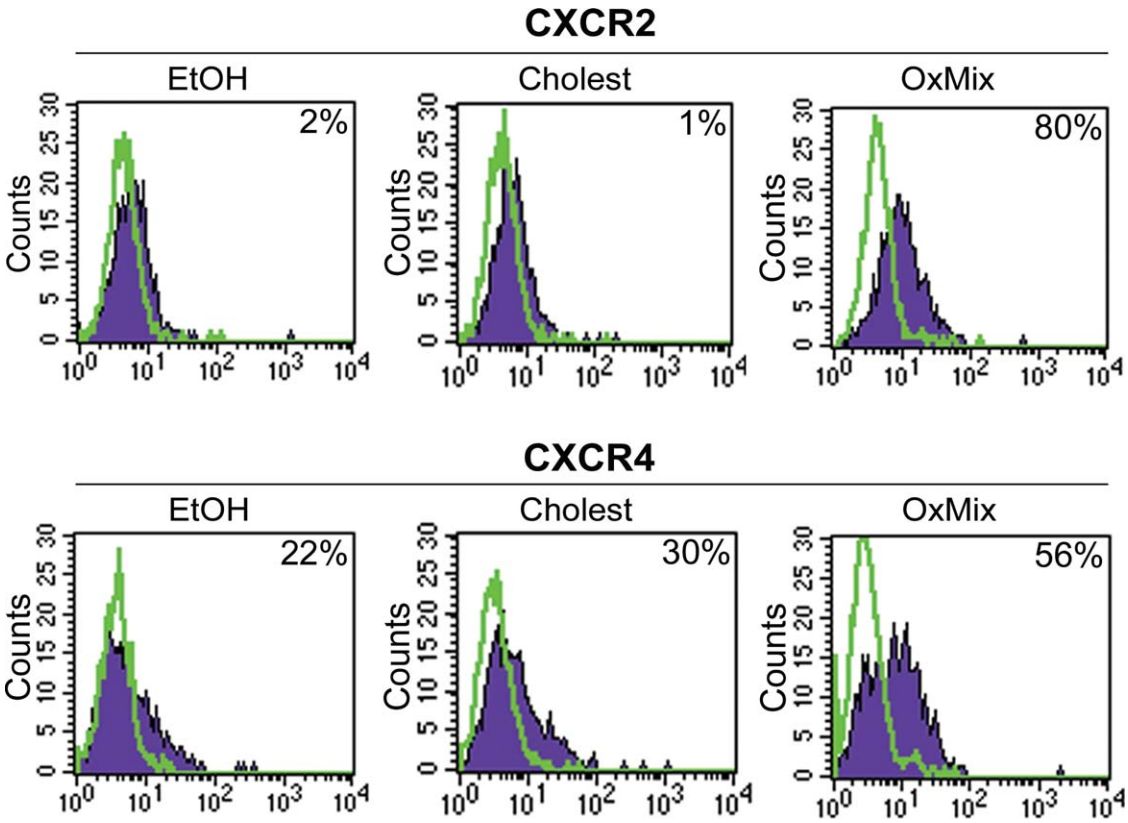

FIG 4

OxMix treatment increases the expression of CXCR2 and CXCR4 on the membrane of human macrophages. CXCR2 and CXCR4 were analyzed by flow cytometry in macrophages treated with ethanol (EtOH), cholesterol (Cholest), or oxysterol mixture (OxMix). The plots shown are representative of five independent experiments carried out on cells isolated from five different donors.

Taking into consideration that OxMix treatment markedly stimulated the IL-10 secretion (Fig. 3A) and reduced the release of MIF (Fig. 3B), the effect of 27-OH on interleukin release was evaluated. As shown in Fig. 6B, a 47% and 34% increase in IL-10 secretion was observed after 8 and 12 H of 27-OH treatment, respectively (Fig. 6B) while no changes in MIF (Fig. 6C), IL-1β (Fig. 6D), and TNF-α (Fig. 6E) release were seen.

Finally, considering that OxMix markedly increased LXRα and ABCA1 expression (Figs. 5A and 5B) and that 27-OH is the major ligand of LXR, we evaluated the impact of 27-OH on the expression of LXR and ABCA1. As shown in Fig. 6F, LXRα mRNA levels were increased in respect to ethanol, 1.5- and 5-fold after 8 and 12 H of treatment, respectively. Moreover, the expression of LXRβ did not change (data not shown). In addition, ABCA1 levels were enhanced 3.5- and 4-fold in respect to ethanol after 8 and 12 H of 27-OH treatment, respectively (Fig. 6G).

4. Discussion

The generation and accumulation of oxysterols within developing plaque, as well as macrophage activation, are important factors modulating the balance of pro- and anti-inflammatory processes and cell survival/apoptosis, responsible for the development of more benign stable lesions or vulnerable plaques [27].

In order to investigate the role of oxysterols in determining the functional polarization of human macrophages, cells were treated with a constant concentration (20 μmol/L) of an OxMix whose composition is similar to that found in advanced human stable carotid plaques [14]. Under this treatment condition, the OxMix induced a decrease in the surface expression of CD36 and CD204, the scavenger receptors known to be crucial for oxysterol uptake by macrophages. Moreover, the OxMix reduced the generation of intracellular ROS levels without affecting cell viability and MMP-9 secretion. Our results agree with a previous study showing that, in apoE$^{-/-}$ mice, the combined deficiency of CD36 and CD204 reduces the formation of advanced atherosclerotic lesions and lowers the inflammatory/oxidative status and apoptosis [28]. Here, the decreased production of ROS found at 12 H is most likely the consequence of the OxMIX-induced M2 macrophage polarization. In fact, as recently demonstrated, M2 polarization decreases the macrophage pro-inflammatory activity by inhibiting the production of ROS [29,30].

According to our results, a single administration of the OxMix (20 μmol/L) did not trigger apoptosis of murine macrophages J774 [31] even though the same treatment produced

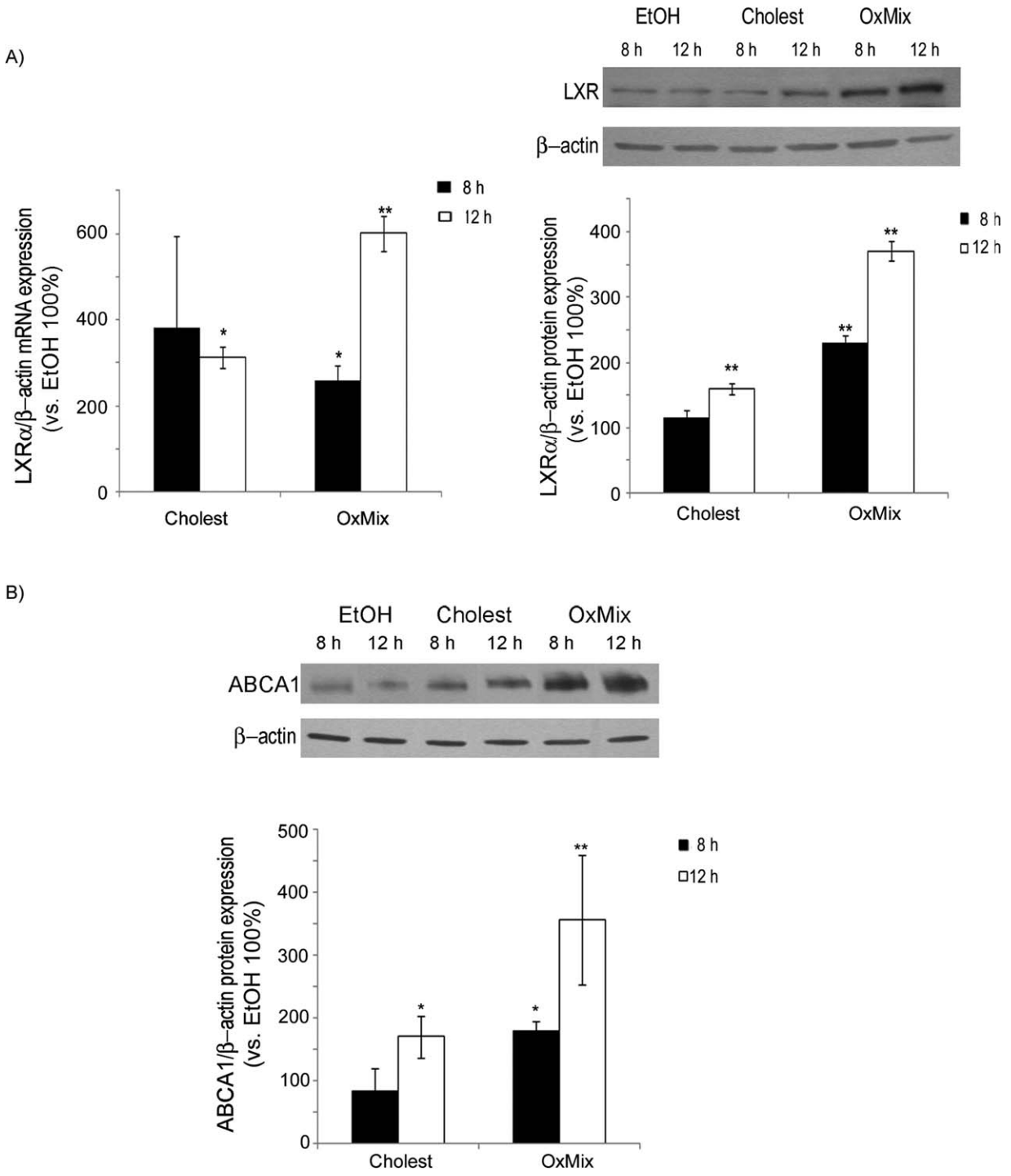

FIG 5

OxMix increases the expression levels of LXRα and ABCA1. ***(A)*** *Real time-PCR (left panel) and western blot (right panel) analysis of LXRα in cholesterol (Cholest) and oxysterol mixture (OxMix)-treated macrophages.* ***(B)*** *Protein levels of ABCA1 in ethanol (EtOH), Cholest, and in OxMix-treated macrophages. Graphed data show mean ± standard deviation from five independent experiments carried out on cells isolated from five different donors. β-Actin is the internal loading control. *P < 0.05 versus EtOH-treated cells **P < 0.01 versus EtOH-treated cells.*

opposite results on ROS production and MMP9 expression in human promonocytic U937 cells [14]. Such a discrepancy is likely due to the different cellular models employed, the different times of incubation and the modality of administration (a single dose of OxMix not followed by other supplies). Moreover, in our experimental model, the OxMix did not induce the

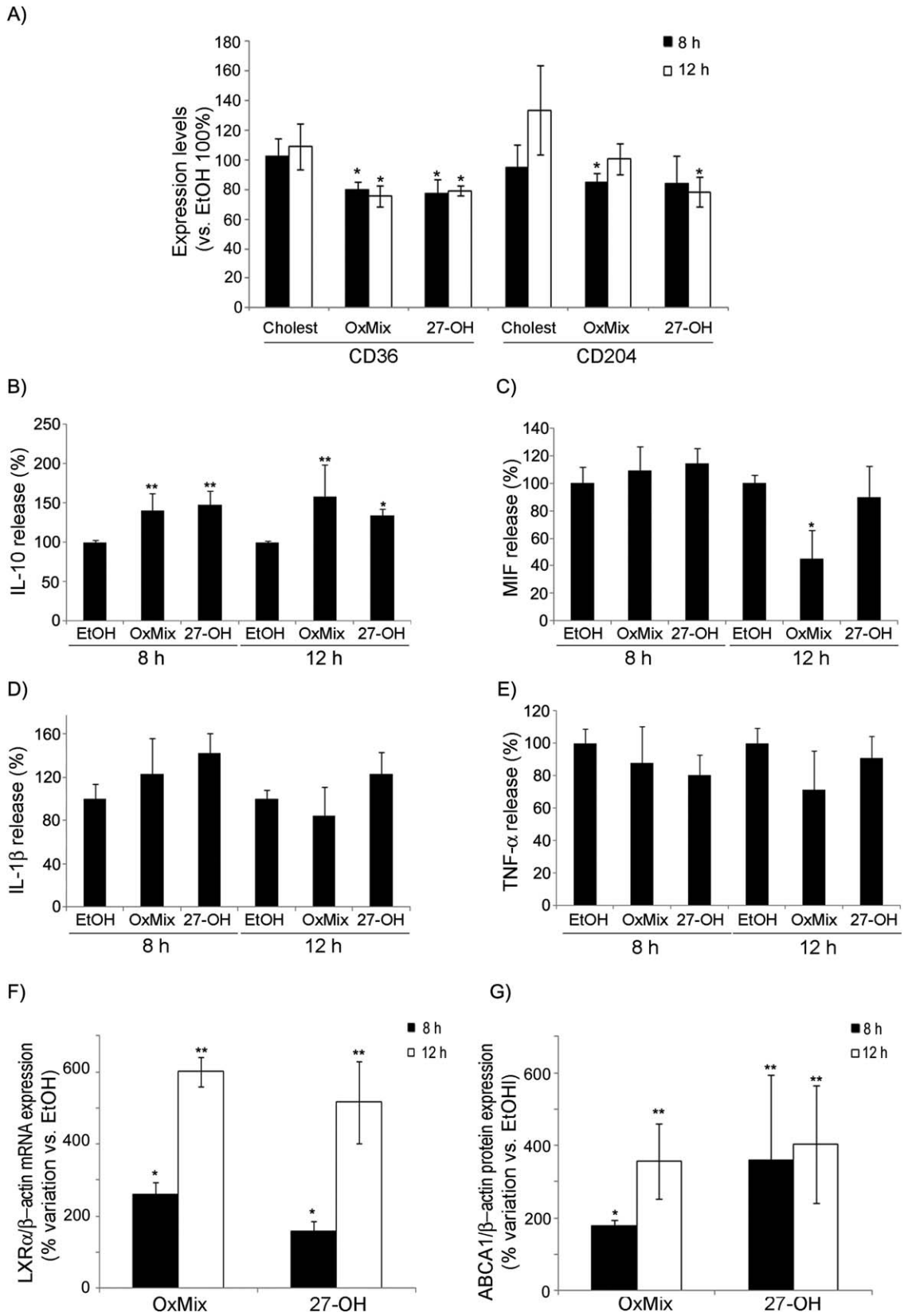

FIG 6

27-OH treatment reduces the levels of CD36 and CD204, stimulates macrophage secretion of IL-10 and increases LXR mRNA and ABCA1 protein levels. ***(A)*** *Surface expression levels of CD36 and CD204 were determined by flow cytofluorimetric analysis and expressed as percentage variation in respect to cells treated with ethanol. The secretion of IL-10* ***(B)****, MIF* ***(C)****, IL-1β* ***(D),*** *and TNF-α* ***(E)*** *was evaluated by ELISA as described in "Materials and Methods" section. Macrophages (M0) were exposed for 8 and 12 H to ethanol (EtOH), oxysterol mixture (OxMix), or 27-OH cholesterol (27-OH).* ***(F)*** *Real time-PCR analyses of LXRα in OxMix and 27-OH treated macrophages.* ***(G)*** *Protein levels of ABCA1 in OxMix- and 27-OH-treated macrophages. The protein levels were expressed as percentage variation in respect to cells treated with ethanol. β-Actin was the internal loading control. Graphed data show mean ± standard deviation from five independent experiments carried out on cells isolated from five different donors. *P < 0.05 versus EtOH-treated cells **P < 0.01 versus EtOH-treated cells.*

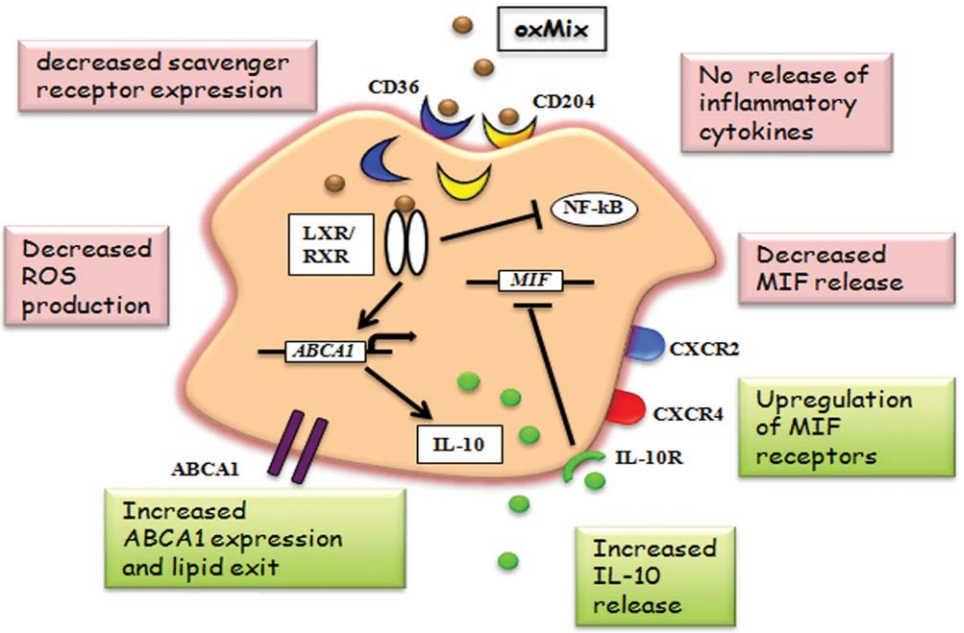

FIG 7

Molecular mechanisms underlying the OxMix-induced M2 polarization. Oxysterol mixture (OxMix) enters the cells directly or via scavenger receptors (CD36 and CD204) and stimulates the expression of Liver-X-receptor (LXR) which, in turn, inhibits inflammation by blocking the NF-kB-dependent pathway [55]. At the same time, LXR markedly increases ATP-binding cassette transporter (ABCA)1 expression which allows the cholesterol efflux [13] and stimulates the secretion of IL-10. This event culminates in a reduction of migration inhibitory factor (MIF) secretion.

expression of surface markers typical of M1 polarization, such as CD80, and the release of pro-inflammatory/immunostimulatory cytokines such as IL-1β, TNF-α, IL-6, IL-18, and CXCL8 (IL-8), but instead stimulated the secretion of the immunomodulatory cytokine IL-10 [32].

Several studies have proposed the anti-atherogenic effect of IL-10 [33–35] even though its protective mechanism against atherogenesis has not yet been fully understood. The IL-10 anti-atherogenic potential seems primarily due to its ability to promote M2 macrophage maturation [32]. Under our experimental conditions, the OxMix did not induce apoptosis probably because of IL-10 release which, in fact, protects against cell death both *in vitro* [36] and *in vivo* [35,37]. Macrophage maturation to M2 phenotype, characterized by IL-10 over-secretion and ROS reduction (Figs. 2C and 3A), is further confirmed by the enhancement of CD204 expression, observed after 12 H of OxMix treatment (Fig. 1B and [38]) while the internalization of the scavenger receptors during the first 8 H of this treatment is likely to be a consequence of oxysterol uptake.

Collectively, our results indicate that oxysterols drive M2 rather than M1 polarization of human macrophages. In particular, the functional phenotype of OxMix-treated macrophages might be similar to that of *in vitro* IL-4-polarized M2a. However, various questions still remain unanswered. These include the ability of this type of macrophages to produce other factors that could influence the inflammatory microenvironment as well as the timing, stability and reversibility of the OxMix-induced polarization [39].

In line with studies in which IL-10 overexpression was reported to inhibit the synthesis of MIF and to reduce the formation of early fatty streaks [34,37], in our study the OxMix decreased MIF release even though it increased the expression of CXCR2 and CXCR4, two receptors capable of binding to MIF, representing a crucial pro-atherogenic factor [40,41].

In our experimental model, the increased surface expression of CXCR2 and CXCR4 detected under OxMix stimulation could reflect a reduced internalization due to the IL-10-mediated reduction of MIF. However, further and more detailed studies are needed to validate such hypothesis, which is not among the aims of this work.

To investigate the macrophage polarizing properties of the OxMix, we analyzed the expression of the oxysterol receptor, LXR, finding that the OxMix treatment induced an increase of both LXR mRNA and protein levels. LXR is the ligand-activated transcription factors of ABCA1 [42] and reduces cholesterol accumulation in macrophages by increasing the ABCA1-dependent cholesterol efflux [43,44]. In line with these observations, ABCA1 expression is increased in OxMix-treated macrophages that release IL-10, a marker of M2 polarization [45]. Interestingly, the activation of LXR has been shown to inhibit the expression of inflammatory genes in atherosclerotic aortas [46].

Twelve-hour treatment with cholesterol also induced an increase of both LXRα and ABC1 and stimulated IL-10 secretion, though at a lower degree compared with the OxMix. On the other hand, cholesterol did not affect CD36, CD204 and CXCR2/4 expression and did not reduce ROS production,

suggesting that, at least in our experimental model, the amount of oxysterols derived from cholesterol oxidation is not sufficient to drive macrophage M2 maturation.

Recently, Buttari and colleagues have demonstrated that 7-oxo-cholesterol skews M2 cell polarization of macrophages toward an M1-like phenotype [47]. In atherosclerotic plaques, however, oxysterols are present as a mixture similar to that used in our study, and never as a single compound. Moreover, 7-oxo-cholesterol does not bind LXR [48] whereas oxysterols, such as 27-OH- and 25-OH-cholesterol, and 5-α-6-α-epoxycholesterol, are potent LXR agonists [49–51].

In our study, 7-oxo-cholesterol represents 21% of the OxMix while 27-OH, 25-OH, and 5-α-6-α-epoxycholesterol represent 28%, 1%, and 8%, respectively. Therefore, 37% of the OxMix components are efficient LXR ligands. In order to assess whether 27-OH is responsible for the observed M2 polarization, human macrophages were treated with 27-OH at the same concentration found in the OxMix. Similarly to the OxMix, 27-OH *per se* induced a marked reduction of CD36 and CD204 levels and stimulated the release of IL-10 by human macrophages. Moreover, 27-OH increased the levels of ABCA1, which is involved in cholesterol efflux and is able to play an anti-atherogenic role. Although we did not directly investigate the activity of LXR, the increased levels of ABCA1, whose gene is a LXR target, makes it reasonable to believe that LXR is activated by 27-OH.

Our results agree with a recent study demonstrating that FTY720, a synthetic sphingosine analogue used in clinical trials as an immunomodulatory drug, confers atheroprotective effects to human macrophages by stimulating the 27-OH/LXR/ABCA1 cascade [52]. In addition, it has been observed that Niemann-Pick C1 $(NPC1)^{-/-}$ macrophages, which have a 27-OH deficiency responsible for a decreased LXR activation and contribute to the formation of atherosclerotic lesions [53]. Furthermore, 27-OH has been found to induce ABCA1 expression by activating the estrogen receptor (ER) [54]. However, the 27-OH-mediated ER activation has also been observed to stimulate pro-inflammatory responses (*e.g.,* IL-1β and TNF-α secretion) [54] which did not occur under our conditions.

Collectively, our results suggest that the M2 macrophage polarization and the anti-inflammatory effects exerted by the OxMix are largely due to the action of 27-OH, the main component of the oxysterol mixture. In this context, a scenario can be envisaged in which oxysterols, and in particular 27-OH, are taken up by macrophages, either directly or *via* scavenger receptors (*i.e.,* CD36 and CD204), with the consequent LXR activation which, in turn, inhibits inflammation by blocking the NF-kB-dependent pathway [55] and by increasing ABCA1 expression, thus stimulating the secretion of IL-10 [45] that reduces MIF release (Fig. 7).

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