Inflammation Research (2019) 68:787–799
https://doi.org/10.1007/s00011-019-01262-8

Inflammation Research

ORIGINAL RESEARCH PAPER

Macrophage lipid accumulation in the presence of immunosuppressive drugs mycophenolate mofetil and cyclosporin A

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Received: 4 March 2019 / Revised: 10 June 2019 / Accepted: 12 June 2019 / Published online: 21 June 2019

Abstract

Objective Mycophenolate (MPA) and cyclosporin A (CsA) are two immunosuppressive agents currently used for the treatment of autoimmune diseases. However, reports regarding their effects on inflammation and lipid handling are controversial. Here, we compare the effect of these two drugs on the expression of proteins involved in cholesterol handling and lipid accumulation in a macrophage cell system utilizing M0, M1 and M2 human macrophages and in murine bone marrow-derived macrophages (BMDM).

Methods Differentiated M0, M1 and M2 subsets of THP-1 human macrophages were subjected to various concentrations of either MPA or CsA. Expression of proteins involved in reverse cholesterol transport (ABCA1 and 27-hydroxylase) and scavenger receptors, responsible for uptake of modified lipids (CD36, ScR-A1, CXCL16 and LOX-1), were evaluated by real-time PCR and confirmed with Western blot. DiI-oxidized LDL internalization assay was used to assess foam cell formation. The influence of MPA was also evaluated in BMDM obtained from atherosclerosis-prone transgenic mice, $ApoE^{-/-}$ and $ApoE^{-/-}Fas^{-/-}$.

Results In M0 macrophages, MPA increased expression of ABCA1 and CXCL16 in a concentration-dependent manner. In M1 THP-1 macrophages, MPA caused a significant increase of 27-hydroxylase mRNA and CD36 and SR-A1 receptor mRNAs. Exposure of M2 macrophages to MPA also stimulated expression of 27-hydroxylase, while downregulating all evaluated scavenger receptors. In contrast, CsA had no impact on cholesterol efflux in M0 and M1 macrophages, but significantly augmented expression of ABCA1 and 27-hydroxylase in M2 macrophages. CsA significantly increased expression of the LOX1 receptor in naïve macrophages, downregulated expression of CD36 and SR-A1 in the M1 subpopulation and upregulated expression of all evaluated scavenger receptors. However, CsA enhanced foam cell transformation in M0 and M2 macrophages, while MPA had no effect on foam cell formation unless used at a high concentration in the M2 subtype.

Conclusions Our results clearly underline the importance of further evaluation of the effects of these drugs when used in atherosclerosis-prone patients with autoimmune or renal disease.

Keywords Immunosuppressive agent · Autoimmune disease · CVD · M1 and M2 subtypes of macrophages · Reverse cholesterol transport · Scavenger receptors · Foam cell formation

Responsible Editor: John Di Battista.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00011-019-01262-8) contains supplementary material, which is available to authorized users.

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Abbreviations

ABC transporters	ATP-binding cassette transporters
BMDM	Bone marrow-derived macrophages
CD36	Cluster of differentiation 36
CsA	Cyclosporin A
CVD	Cardiovascular disease
CXCL16	Chemokine CXC ligand 16
DMSO	Dimethyl sulfoxide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
LDL	Low-density lipoprotein

LOX-1	Lectin-like oxidized low-density lipoprotein receptor
MPA	Mycophenolate mofetil
PMA	Phorbol 12-myristate 13-acetate
ScR-AI	Scavenger receptor class A type I
SLE	Systemic lupus erythematosus

Introduction

Sustained inflammation in systemic lupus erythematosus (SLE) patients leads to accelerated atherosclerosis. On the other hand, nephrotic syndrome and proteinuria, conditions that commonly develop in SLE patients, add to induction of an adverse lipid profile and higher pro-thrombotic risk. A unique combination of atherosclerotic and thrombo-embolic events contributes to elevated risk of cardiovascular disease (CVD) in SLE patients [1]. Thus, SLE patients display a five- to ninefold increase in incidence of coronary heart disease compared to individuals of the same age with no SLE, and similar risk factors [2].

Common therapeutic interventions in SLE include immunosuppressive drugs such as mycophenolate mofetil (MPA) and cyclosporin A (CsA) [3, 4]. Despite similar efficacy in immunosuppression, MPA and CsA differ in regulation of lipid accumulation and atherosclerosis progression in vivo and in vitro.

Thus, MPA has been reported to inhibit expression of adhesion molecules [5], suppress nitric oxide production [6], activate cholesterol efflux in vitro [7] and, therefore, reduce progression of atherosclerosis in animal models [8–11]. In contrast to MPA, reports on the effect of CsA on lipid handling and atherosclerosis progression are controversial. Earlier studies in animal models indicated anti-atherogenic features of CsA [12]. However, in later investigations, utilizing mouse and rabbit models, CsA has been shown to promote atherosclerosis, primarily affecting reverse cholesterol transport [13].

Reports on clinical outcomes of MPA and CsA treatment of SLE patients are controversial as well. Zahr et al. [14] and van Leuven [15] indicated a reduction of atherosclerosis and immunological disease activity in lupus patients upon MPA treatment in a concentration-dependent manner. In contrast, in a cross-sectional analysis, when MPA treatment was compared with other treatment options for SLE patients (azathioprine or methotrexate), Kaini et al. [16] and McMahon et al. [17] found no difference in the baseline prevalence of either cardiovascular disease or lupus progression.

CsA has a strong record of inhibiting cholesterol efflux in murine macrophages leading to increased foam cell formation [18]. However, recent studies demonstrated a beneficial influence of CsA on atherosclerosis in CVD and autoimmune diseases such as rheumatoid arthritis [19, 20] and SLE [21].

Therefore, this study was conducted to investigate the effect of these two drugs on lipid handling in an identical setting utilizing the human THP-1 macrophage cell system, a widely used cell culture model for macrophage behavior in response to conditions mimicking the atherosclerotic process in vitro [22]. Moreover, these effects were compared in the three functional classifications of macrophages: M0, M1 and M2 subtypes to replicate the different populations of macrophages present in healthy individuals and patients with autoimmune diseases [23]. Finally, effects of MPA were evaluated in BMDM from atherosclerosis-prone mice with (double knockout ApoE$^{-/-}$Fas$^{-/-}$) and without (single knockout ApoE^{-}/$^{-}$) a lupus-like disease [24].

Methods

Cells and reagents

THP-1 monocytes were purchased from the American Type Culture Collection (Manassas, VA). Cell culture media (RPMI 1640), supplementary reagents and Trizol reagent were obtained from Invitrogen (Carlsbad, CA).

Lipopolysaccharides (LPS) from *Escherichia coli,* Phorbol 12-myristate 13-acetate (PMA), cyclosporine A and mycophenolic acid (MPA)—6-(1,3-dihydro-7-hydroxy-5-methoxy-4-methyl-1-oxoisobenzofuran-6-yl)-4-methyl-4-hexanoic acid, and 6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid were obtained from Sigma-Aldrich (St. Louis, MO).

Oxidized low-density lipoproteins (oxidized LDL) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylin docarbocyaninet (DiI)-oxidized LDL were purchased from Intracel (Frederick, MD).

All reagents for QRT-PCR and interferon-γ (IFN-γ) were acquired from Roche Applied Science (Indianapolis, IN). All reagents for total protein extraction and Western blot analysis were obtained from ProteinSimple (Santa Clara, CA).

Cell culture

The THP-1 human monocytic leukemia cell line was chosen for the study because it shares many properties with normal human monocytes, including expression of scavenger receptors and cholesterol transport proteins, and is a well-accepted model for atherosclerosis used in our laboratory and many others [22].

THP-1 monocytes were grown at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 units/ml streptomycin. To facilitate differentiation into macrophages, THP-1

monocytes were treated with 100-nM PMA for 24 h at 37 °C. When a differentiated non-polarized phenotype (M0) was achieved, the PMA-containing medium was removed, and replaced with complete RPMI 1640 supplemented with 10% fetal calf serum for another 24 h.

To generate polarized phenotypes, cells were then cultured for 48 h in the presence of 20 ng/ml IFN-γ and 100 ng/ml LPS to obtain M1 macrophages [25] or 20 ng/ml interleukin (IL)-4 to obtain M2 macrophages [26]. Phenotypes were confirmed via QRT-PCR analysis (Suppl. Figure 1) of gene expression of commonly used cell surface markers IL-12, CCR7, and CXC chemokine ligand (CXCL)10 for M1 and CD163, mannose receptor, and CCL17 for M2 (Table 1).

Phenotypic changes in macrophages were verified by flow cytometry using a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ). Both M1 and M2 phenotypes were stained with a PE-conjugated monoclonal antibody to CD163, a FITC-conjugated monoclonal antibody to CD68, an APC-conjugated monoclonal antibody to CD11b, and a PE/Cy7-conjugated monoclonal antibody to CD14, as well as isotype-matched control monoclonal antibodies, for 30 min at room temperature according to the manufacturer's instructions (Abcam, Cambridge, MA) [23, 25–27].

For BMDM studies, the use of mice was approved by the NYU Winthrop Hospital IACUC. Femurs were dissected and cell suspensions obtained by flushing femurs and tibias with sterile phosphate-buffered saline (PBS). After dispersion by passing the marrow through an 18-gauge needle several times, cells were centrifuged at 500×*g* for 5 min. Cells were then resuspended in R10 medium containing 15% L929-cell conditioned medium, plated in 100-mm culture dishes, and incubated at 37 °C in a 5% CO2 atmosphere for 7 days. Medium was aspirated and replaced on days 3 and 5. BMDM were generated by culturing cells with MCSF medium for 7 days. Cells from all genotypes of mice: C57BL/6J, ApoE$^{-/-}$ and ApoE$^{-/-}$Fas$^{-/-}$ [28] were subjected to the same experimental conditions, except CsA.

Table 1 Macrophage phenotype markers used for QRT-PCR

Gene	Primer
IL-12	F 5′-AAAGGACATCTGCGAGGAAAGTTC-3′ R 5′-CGAGGTGAGGTGCGTTTATGC-3′
CCR7	F 5′-TGGTGGTGGCTCTCCTTGTC-3′ R 5′-TGTGGTGTTGTCTCCGATGTAATC-3′
CXCL10	F 5′-GAAAGCAGTTAGCAAGGAAAGGTC-3′ R 5′-ATGTAGGGAAGTGATGGGAGAGG-3′
CD163	F 5′-GTCGCTCATCCCGTCAGTCATC-3′ R 5′-GCCGCTGTCTCTGTCTTCGC-3′
Mannose receptor	F 5′-ACCTCACAAGTATCCACACCATC-3′ R 5′-CTTTCATCACCACACAATCCTC-3′
CCL17	F 5′-CGGGACTACCTGGGACCTC-3′ R 5′-CCTCACTGTGGCTCTTCTTCG-3′

Experimental conditions

Upon achieving M0, M1 and M2 phenotype, macrophages were cultured for an additional 24 h prior to treatment. Macrophages were incubated in 12-well plates for 18 h under the following conditions: (1) RPMI medium alone; (2) ethanol solvent control; (3) dimethyl sulfoxide (DMSO) solvent control; (4) 0.5 μg/ml MPA; (5) 1 μg/ml MPA; (6) 5 μg/ml MPA; (7) 1 μg/ml CsA; (8) 5 μg/ml CsA (foam cell formation only).

RNA isolation and QRT-PCR

Immediately after the incubation period, total RNA was isolated with Trizol (Carlsbad, CA) reagent (1 ml/10^6 cells). The quantity of total RNA from each condition was measured by absorption at 260-nm and 280-nm wavelengths by ultraviolet spectrophotometry (Hitachi U2010 spectrophotometer).

ATP binding cassette transporter (ABC) A1, 27-hydroxylase, lectin-like oxidized LDL receptor (LOX)1, scavenger receptor (SR)-A1, CXCL16, CD36. mRNA were quantified by real-time PCR. The cDNA was copied from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase primed with oligo dT. Equal amounts of cDNA were taken from each RT reaction mixture for PCR amplification using specific primers (Table 2). QRT-PCR analysis was performed using the SYBR Green Reagent Kit according to the manufacturer's instructions on the Roche Light Cycler 480. Each reaction was done in triplicate. The amount of PCR products were estimated using Roche Applied Science software, provided by the manufacturer. Fluorescence emission spectra were monitored and analyzed. PCR products were measured by the threshold cycles (CT), at which specific fluorescence becomes detectable. The CT value

Table 2 The list of specific primers used for QRT-PCR

Gene	Primer
ABCA1	F 5′-GAAGTACATCAGAACATGGGC-3′ R 5′-GATCAAAGCCATGGCTGTAG-3′
27-hydroxylase	F 5′-AAGCGATACCTGGATGGTTG-3′ R 5′-TGTTGGATGTCGTGTCCACT-3′
CD36	F 5′-GAGAACTGTTATGGGGCTAT-3′ R 5′-TTCAACTGGAGAG-GCAAAGG-3′
LOX-1	F 5′-TTACTCTCCATGGTGGTGCC-3′ R 5′-AGCTTCTTCTGCTTGTTGCC-3′
ScR-A1	F 5′-CTCGTGTTTGCAGTTCTCA-3′ R 5′-CCATGTTGCTCATGTGTTCC-3′
CXCL16	F 5′-ACTACACGACGTTCCAGCTCC-3′ R 5′-CTTTGTCCGAGGACAGTGATC-3′
GAPDH	F 5′-ACCATCATCCCTGCCTCTAC-3′ R 5′-CCTGTTGCTGTAGCCAAAT-3′

for each gene was normalized to that of glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The fold change in expression level of target gene relative to the GAPDH at various time points was calculated using $2^{-\Delta\Delta CT}$ method [29]. The relative expression level was calculated as the mean value of the untreated THP-1 as 1. Non-template controls were included for each primer pair to check for significant levels of any contaminants. A melting-curve analysis was performed to assess the specificity of the amplified PCR products.

Protein extraction and Western Blot analysis

Cellular extracts were prepared with Lysis Kit—RIPA Buffer (ProteinSimple, Santa Clara, CA). The immunoreactive proteins were detected using Wes Assay kit (ProteinSimple, Santa Clara, CA).

Rabbit anti-human ABCA1 (sc-20,794) (Santa Cruz, CA) was used as primary antibodies for detection of ABCA1. Anti-cholesterol 27-hydroxylase antibody is an affinity-purified rabbit polyclonal antibody raised against residues 15–28 of the cholesterol 27-hydoxylase protein [30]. Rabbit anti-human LOX1 (ab60178), CD36 (ab64014), SR-A1 (ab36625), and CXCL16 (ab101404) antibodies were purchased from Abcam Inc. (Cambridge, MA). As a loading control, GAPDH was detected using rabbit anti-human GAPDH antibody (ab9485).

Quantization of detected proteins and image preparation were performed with Compass Software (ProteinSimple, Santa Clara, CA).

For blotting studies, protein samples (20 µg/lane) were boiled for 5 min, and fractionated on 8% SDS–PAGE, and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was stained with Ponceau red (Sigma, St. Louis, MO) to verify uniformity of protein loading in each lane. The membrane was blocked for 1 h at room temperature in blocking solution (3% nonfat dry milk (Bio-Rad) in 1X Tris-buffered saline/1% Tween 20 [TTBS]) and then immersed in a 1:500 dilution of primary antibody overnight at 4 °C. The following day, the membrane was washed and then incubated in a 1:5000 dilution of ECL horseradish peroxidase-linked species-specific whole antibody in blocking solution.

The immunoreactive proteins then were detected using Pierce ECL Western Blot substrate system, and film development in SRX-101A (Konica Minolta Holdings, Inc., Tokyo, Japan). Stripping and reprobing of the membranes were performed according to the manufacturer's protocol (ECL kit instructions, Thermoscientific, Rockford, IL). Band intensities for Western blot protein samples were quantified using Kodak Digital Science 1D, version 2.0.3, after imaging with Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

Evaluation of foam cell formation and quantization of lipid accumulation by DiI-oxidized LDL internalization assay

Foam cell transformation was evaluated in all subtypes of macrophages based on two different approaches conducted simultaneously. THP-1-differentiated macrophages were cultured under the same conditions described in the "Experimental conditions" section in the presence of 50 µg/ml oxidized LDL (oxLDL) for 24 h. After incubation, cells were subjected to two analyses: Oil-red-O staining of macrophages and DiI-oxLDL internalization assay.

For the foam cell formation assay, cells were washed in distilled water and stained with 0.2% Oil Red O (Sigma, St. Louis, MO) for 30 min. After the PBS wash, cell nuclei were stained with hematoxylin (Sigma, St. Louis, MO) for 5 min. After a final wash with PBS, coverslips were mounted on slides using Permount solution (Sigma, St. Louis, MO). Foam cells, recognized as red-stained cells, were visualized via light microscopy (Axiovert 25; Carl Zeiss, Gottingen, Germany) with 40× magnification and photographed using a DC 290 Zoom digital camera (Eastman Kodak, Rochester, NY).

For the DiI-oxLDL internalization assay, cells were subjected to the same conditions as described above followed by 5 µg/ml (DiI)-oxLDL for 4 h. Slides were prepared using Vectashield mounting medium containing DAPI stain (Vector Laboratories, Inc., Burlingame, CA). After incubation, accumulation of DiI-oxLDL in cells was determined by fluorescent intensity with a Nikon A1 microscopy unit with 40× magnification and photographed with a DS-Ri1 digital camera. Fluorescent intensity was quantified from at least 3 random fields (1024 × 1024 pixels) per slide, from three slides per experimental condition and graphed.

Statistical analysis of experimental data

Statistical analysis was performed using Graphpad Prism, version 5.01 (GraphPad Software, San Diego, CA). All data were analyzed by one-way analysis of variance, and pairwise multiple comparisons were made between control and treatment conditions using Bonferroni correction. Probability values less than 0.05 were regarded as significant.

Results

MPA and CsA and the expression of cholesterol efflux proteins in different subtypes of macrophages

In all our experiments, levels of mRNA in all subpopulations of THP-1 macrophages, incubated with solvent control (ethanol for MPA and DMSO for CsA), were set at 100%.

mRNA expression analysis was supported by corresponding Western blot.

Exposure of naïve M0 macrophages to different concentrations of MPA revealed a concentration-dependent rise in the expression of the ABCA1 transporter (Fig. 1a, d). Thus, incubation of M0 THP-1 macrophages with 0.5 µg/ml MPA elevated ABCA1 mRNA to 108.99 ± 22.80% and with 1 µg/ml MPA to a level of 152.02 ± 30.86% vs. solvent control ($n = 3$, NS). Application of 5 µg/ml MPA upregulated ABCA1 message to 183.31 ± 22.91% vs. solvent control ($n = 3$, $P < 0.01$) set at 100%. Conversely, incubation of THP-1-naïve macrophages (M0) in 1 µg/ml CsA did not significantly alter expression of the ABCA1 transporter (Fig. 1a, d). Neither MPA, nor CsA affected the expression of 27-hydroxylase, indicating that the change in ABCA1 was 27-hydroxylase independent.

Incubation of M1 macrophages with the identical concentrations of MPA surprisingly caused an opposite effect (Fig. 1b, d). Thus, expression of ABCA1 did not significantly change at either the mRNA or protein level. However, expression of 27-hydroxylase was significantly upregulated, reaching 185.07 ± 26.74% ($n = 3$, $P < 0.05$) at 1 µg/ml MPA

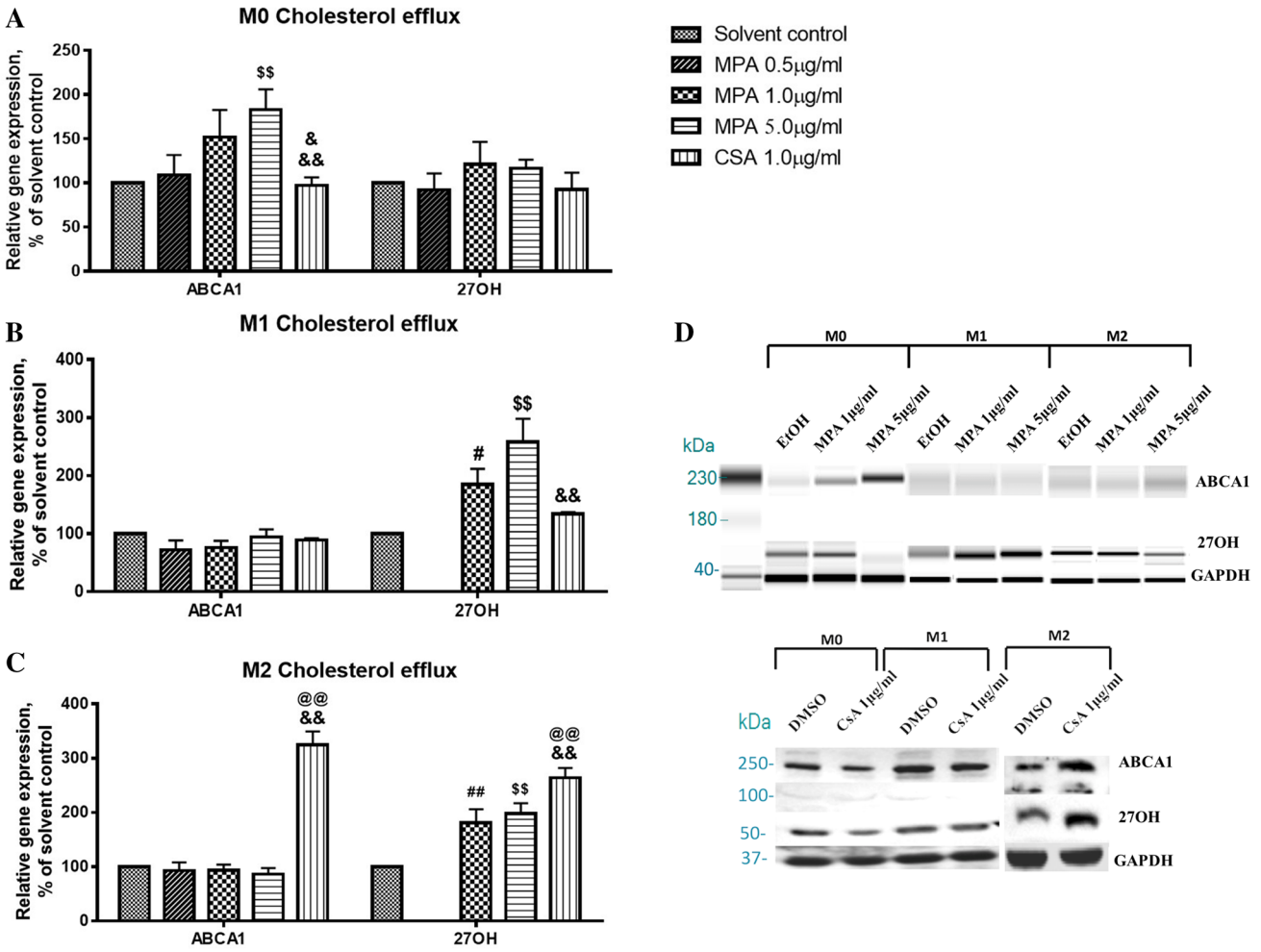

Fig. 1 Effect of MPA and CsA on the expression of cholesterol efflux proteins in THP-1 macrophages. M0, M1 and M2 subpopulations of human THP-1 macrophages were subjected to different concentrations of MPA and CsA, as indicated in the "Methods" section. Following incubation, mRNA was isolated for each condition, reverse transcribed and amplified by QRT-PCR with GAPDH message as an internal standard. Gene expression levels were calculated and graphed as change vs. solvent control set at 100%. In naïve THP-1 macrophages **a** MPA stimulated a concentration-dependent increase in the expression of the ABCA1 transporter, while no effect was detected in the presence of CsA. Neither MPA, nor CsA affected the expression of 27-hydroxylase (27OH) (**a**). MPA and CsA had no significant influence on the expression of ABCA1, but increased 27OH mRNA in M1 macrophages (**b**). In the M2 subpopulation of macrophages, MPA alters cholesterol efflux augmenting the expression of 27OH, but not ABCA1. CsA significantly enhances expression of both ABCA1 and 27OH (**c**). Gene expression analysis was confirmed by Western blot (**d**). Values are mean ± SEM of three independent experiments. $$, **, @@, ##$P < 0.01$ vs. solvent control in M0, M1 and M2, correspondingly; &$P < 0.05$ vs. 1 µg/ml MPA; &&$P < 0.01$ vs. MPA 5 µg/ml

and 258.46 ± 39.81% ($n = 3$, $P < 0.01$) at 5 μg/ml MPA (Fig. 1b). M1-differentiated macrophages exposed to CsA behaved similarly to M0, exhibiting no significant changes in the expression of key cholesterol efflux proteins (Fig. 1b, d).

A different response was detected when M2-differentiated macrophages were subjected to the same conditions (Fig. 1c, d). Similar to the M1 population of macrophages, MPA had no effect on the expression of the ABCA1 transporter. Conversely, incubation of this subpopulation of macrophages with 1 μg/ml CsA resulted in more than a threefold elevation of ABCA1 mRNA versus solvent control to 325.27 ± 24.20% ($n = 3$, $P < 0.01$) (Fig. 1c, d). Both drugs had a substantial impact on 27-hydroxylase mRNA expression in M2 macrophages. Thus, MPA caused a concentration-dependent upregulation of 27-hydroxylase mRNA to 180.67 ± 25.58% at 1 μg/ml ($n = 3$, $P < 0.01$) and 198.57 ± 18.56% at 5 μg/ml ($n = 3$, $P < 0.01$) vs. solvent control (Fig. 1c). However, we did not observe significant changes on the protein level (Fig. 1d). Exposure of M2 macrophages to 1 μg/ml CsA caused in excess of double the mRNA level of 27-hydroxylase compared to the corresponding solvent control ($n = 3$, $P < 0.01$). Results were confirmed with Western blot (Fig. 1c, d).

MPA and CsA and the expression of scavenger receptors, involved in cholesterol influx in different subtypes of macrophages

MPA had no effect on the expression of major scavenger receptors responsible for lipid uptake: CD36, SR-A1 and LOX-1 in naïve THP-1 macrophages (Fig. 2a, d). The level of CXCL16 mRNA was increased to 152.78 ± 33.01% of solvent control upon exposure of M0 macrophages to 5 μg/ml MPA ($n = 3$, $P < 0.05$).

Incubation of M0 macrophages with 1 μg/ml CsA did not change expression of CD36 and SR-A1, as well as CXCL16 (Fig. 2a). However, LOX-1 was significantly increased to 120.15 ± 4.80% when subjected to the same concentration of CsA ($n = 3$, $P < 0.05$).

An interesting response in the expression of scavenger receptors was observed upon exposure of M1 macrophages to both immunosuppressive agents—MPA and CsA (Fig. 2b, d). MPA significantly augmented CD36 mRNA, increasing the message to 194.88 ± 8.12% at 0.5 μg/ml, to 181.66 ± 12.45% at 1 μg/ml and to 241.52 ± 31.77% at 5 μg/ml ($n = 3$, $P < 0.01$). Conversely, CsA significantly downregulated expression of the CD36 scavenger receptor, reducing it to 78.19 ± 2.54% versus solvent control set at 100% ($n = 3$, $P < 0.01$).

Changes in the level of SR-A1 showed the same pattern (Fig. 2b, d). Thus, the presence of MPA increased SR-A1 mRNA to 207.63 ± 32.37% at 0.5 μg/ml, to 150.69 ± 14.13% at 1 μg/ml and to 188.72 ± 24.48% at 5 μg/ml ($n = 3$, $P < 0.01$). Incubation with CsA had the opposite effect and led to a decrease in the expression of this scavenger receptor to 79.48 ± 8.24% versus solvent control ($n = 3$, $P < 0.05$).

The levels of LOX1 and CXCL16 mRNA in M1 phenotype macrophages were not affected by the presence of either MPA or CsA (Fig. 2b, d).

Exposure of M2-differentiated macrophages to MPA had no significant effect on the mRNA level of CD36 or CXCL16 (Fig. 2c). In contrast, MPA significantly downregulated expression of SR-A1 and LOX1 scavenger receptors. Thus, SR-A1 message was reduced to 69.87 ± 16.47% upon incubation of M2 macrophages with 0.5 μg/ml MPA ($n = 3$, $P < 0.05$). It remained suppressed at similar levels when cells were exposed to increased concentrations of MPA [74.12 ± 8.25% at 1 μg/ml and 68.17 ± 12.65% at 5 μg/ml ($n = 3$, $P < 0.05$)]. Level of LOX-1 mRNA was decreased to 47.12 ± 11.29% at 0.5 μg/ml MPA, 42.41 ± 4.72% at 1 μg/ml MPA, and 33.40 ± 3.46% at 5 μg/ml ($n = 3$, $P < 0.0001$). Surprisingly, incubation of M2 phenotype macrophages with CsA caused increased expression of all investigated scavenger receptors (Fig. 2c, d). Thus, CD36 was elevated to 195.81 ± 3.17% ($n = 3$, $P < 0.01$), SR-A1—to 157.78 ± 6.17% ($n = 3$, $P < 0.01$), LOX-1—to 162.25 ± 2.49% ($n = 3$, $P < 0.01$) and CXCL16—to 173.68 ± 3.06% ($n = 3$, $P < 0.01$).

Alterations in DiI-oxLDL uptake and foam cell transformation in the presence of MPA and CsA in cultured THP-1 macrophages

Next, we examined the effect of MPA and CsA on foam cell formation in different phenotypes of THP-1 macrophages. In these experiments, concentration of CsA at 5 μg/ml was included for a better comparison with MPA. After incubation, cells were subjected to two examinations: Oil-red-O staining of macrophages and DiI-oxLDL internalization assay, as described in "Methods" section.

The results of the in DiI-oxLDL internalization analysis demonstrated no significant changes in LDL uptake in M0 and M2 macrophages, caused by MPA or CsA (Fig. 3a). The only significant increase in LDL uptake was detected in M1 macrophages when these cells were exposed to 1 μg/ml MPA, reaching 178.8 ± 18.5% ($n = 5$, $P < 0.05$) (Fig. 3a). However, at 5 μg/ml MPA, oxLDL accumulation was not different from the solvent control set at 100%, and its value was significant in comparison to the lower concentration of 1 μg/ml MPA ($n = 5$, $P < 0.01$). The microphotographs of DiI-oxLDL internalization (supplementary Fig. 2) and oil-red-O staining of LDL (Fig. 3) depict lipid uptake in M0 (B), M1 (C) and M2 (D) macrophages exposed to MPA and CsA.

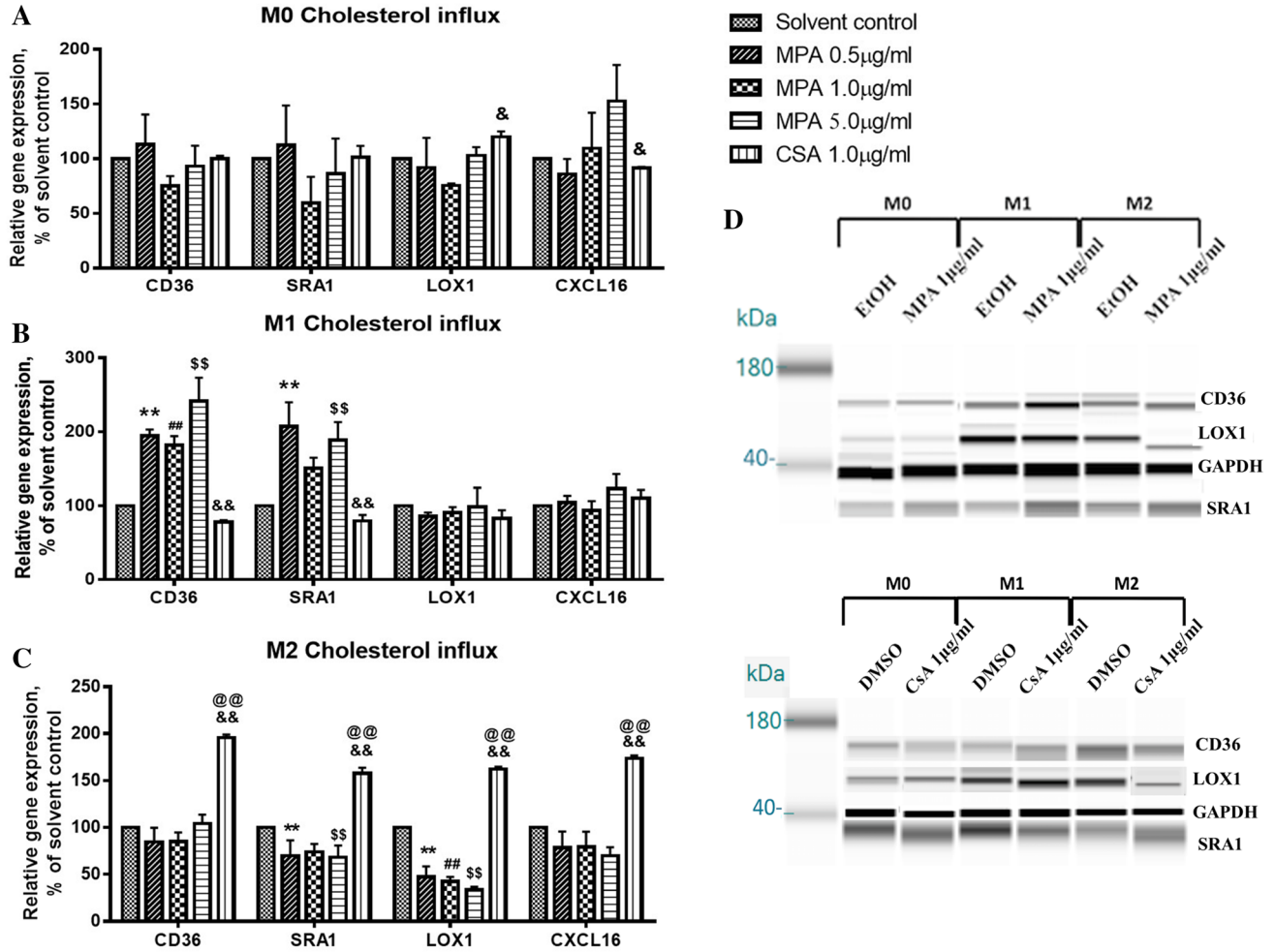

Fig. 2 The expression of scavenger receptors in THP-1 macrophages in the presence of MPA and CsA. M0, M1 and M2 subpopulations of human THP-1 macrophages were subjected to different concentrations of MPA and CsA, as indicated in the "Methods" section. Following incubation, mRNA was isolated for each condition, reverse transcribed and amplified by QRT-PCR with GAPDH message as an internal standard. Gene expression levels were calculated and graphed as change vs. solvent control set at 100%. Exposure of M0 macrophages to MPA upregulated the expression of CXCL16, while the presence of CsA augments the expression of LOX1 (**a**). In M1 macrophages, MPA significantly enhances mRNA level of the main scavenger receptors involved in oxLDL uptake—CD36 and SR-A1. CsA has an opposite effect—decreasing the expression of CD36 and SR-A1 (**b**). MPA downregulates expression of CD36 and SR-A1 in the M2 subpopulation of macrophages. Application of CsA caused an augmentation of all evaluated scavenger receptors in this subpopulation of macrophages (**c**). Gene expression analysis was confirmed by Western blot (**d**). Values are mean ± SEM of three independent experiments. **, ##, $$, @@ $P < 0.01$ vs. solvent control, && $P < 0.05$ vs. 1 μg/ml (or 5 μg/ml) MPA, && $P < 0.01$ 1 μg/ml (or 5 μg/ml) MPA

Effect of MPA on the expression of proteins involved in lipid accumulation in BMDM from WT, ApoE$^{-/-}$ and ApoE$^{-/-}$/Fas$^{-/-}$ mice

As a proof of concept, we have conducted a series of experiments in BMDM obtained from mice with C57BL/6J (wild type), ApoE$^{-/-}$ (a common knockout mouse model of atherosclerosis) and ApoE$^{-/-}$Fas$^{-/-}$ (double knockout mouse with atherosclerosis and lupus-like disease) [24], using similar experimental conditions for MPA. The first set contained gene expression analysis consisting of the three key genes involved in lipid accumulation: ABCA1, CD36 and SR-A1. The second set included the evaluation of lipid accumulation by oil-red-O and DiI-LDL internalization assays.

We observed a decrease in the expression of the ABCA1 transporter upon exposure of BMDM to MPA in all genotypes of mice ($n = 3$, $P < 0.01$) (Fig. 4a). However, no changes were detected at the protein level (Fig. 4b).

MPA application did not produce significant changes in the level of CD36 mRNA in macrophages from wild-type and lupus-like mice (Fig. 4a). However, in BMDM from ApoE$^{-/-}$ mice, MPA stimulated a concentration-dependent increase in the expression of scavenger receptor CD36 vs. solvent control set at 100%, reaching maximum upregulation

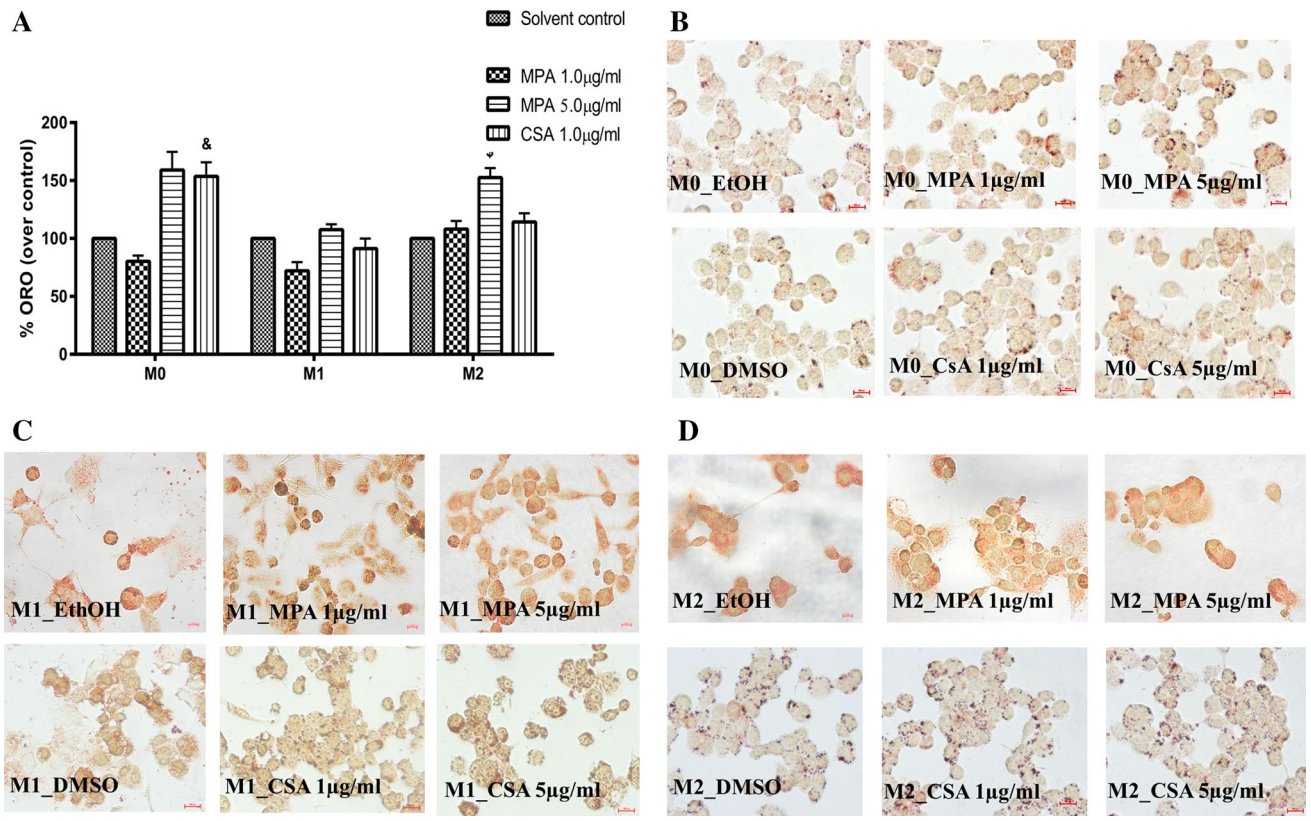

Fig. 3 Foam cell transformation induced by MPA and CsA in different subpopulations of THP-1 macrophages. M0, M1 and M2 subpopulations of macrophages were subjected to different concentrations of MPA and CsA in the presence of oxLDL for 24 h. For LDL internalization assay and foam cell quantization, the treatment was followed by incubation with DiI-oxLDL for 4 h. Washed cells were covered with DAPI-containing medium, allowing staining of the nuclei. Fluorescent intensity was determined with a Nikon A1 microscopy unit (×40) and photographed with a DS-Ri1 digital camera. Fluorescent intensity was quantified from three random fields (1024 × 1024 pixels) per slide, from three slides per experimental condition ($n = 9$) and graphed (**a**). Foam cell transformation was visualized by oil-red-O staining of oxLDL in each type of macrophage and presented as photomicrographs done with a DS-Ri1 digital camera (×40) (**b–d**). Values are mean ± SEM of three independent experiments. ##$P < 0.01$ vs. solvent control; &&$P < 0.01$ vs. 1 μg/ml MPA

of mRNA at 5 μg/ml MPA—153.7 ± 30.7% ($n = 3$, $P < 0.05$) (Fig. 4a). This increase was confirmed on the protein level (Fig. 4b).

The SR-A1 mRNA was not significantly changed in BMDM from all genotypes of mice in the presence of MPA (Fig. 4a). We observed a peculiar pattern in response to MPA application in BMDM from atherogenic and lupus mice. Thus, expression of SR-A1 was increased in the presence of 0.5 μg/ml MPA followed by decrease to the level of control at 1 μg/ml and 5 μg/ml. The same pattern appeared even when experiments were repeated three times in triplicates.

Next, we examined the accumulation of lipids in BMDM in the presence of MPA using oil-red-O staining (Fig. 4c) and DiI-oxLDL internalization analysis for quantization of LDL uptake (Suppl. Figure 3). Overall, MPA had an impact on LDL accumulation only in BMDM from ApoE$^{-/-}$ when higher concentration (5 μg/ml) was used (compared to solvent control). Thus, this concentration caused an increase in DiI-LDL uptake to 165.7 ± 28.9% versus solvent control set at 100% ($n = 6$, $P < 0.05$). All other conditions did not display a significant difference.

Discussion

CVD is strongly linked with chronic inflammation, and a major cause of mortality in patients with autoimmune diseases, including SLE [31]. Our group has published multiple reports indicating that pro-inflammatory cytokines such as IFN-γ and TNF-α that are found in the autoimmune state are pro-atherogenic, encouraging an imbalance between cholesterol influx and efflux [32, 33]. Recent reports indicate that release of these cytokines leads to accumulation of M1 macrophages and further promotes clinical manifestations of SLE [34, 35]. Moreover, the presence of M1 macrophages has been found in progressing atherosclerotic plaques. Conversely, the alternatively activated M2 phenotype has been

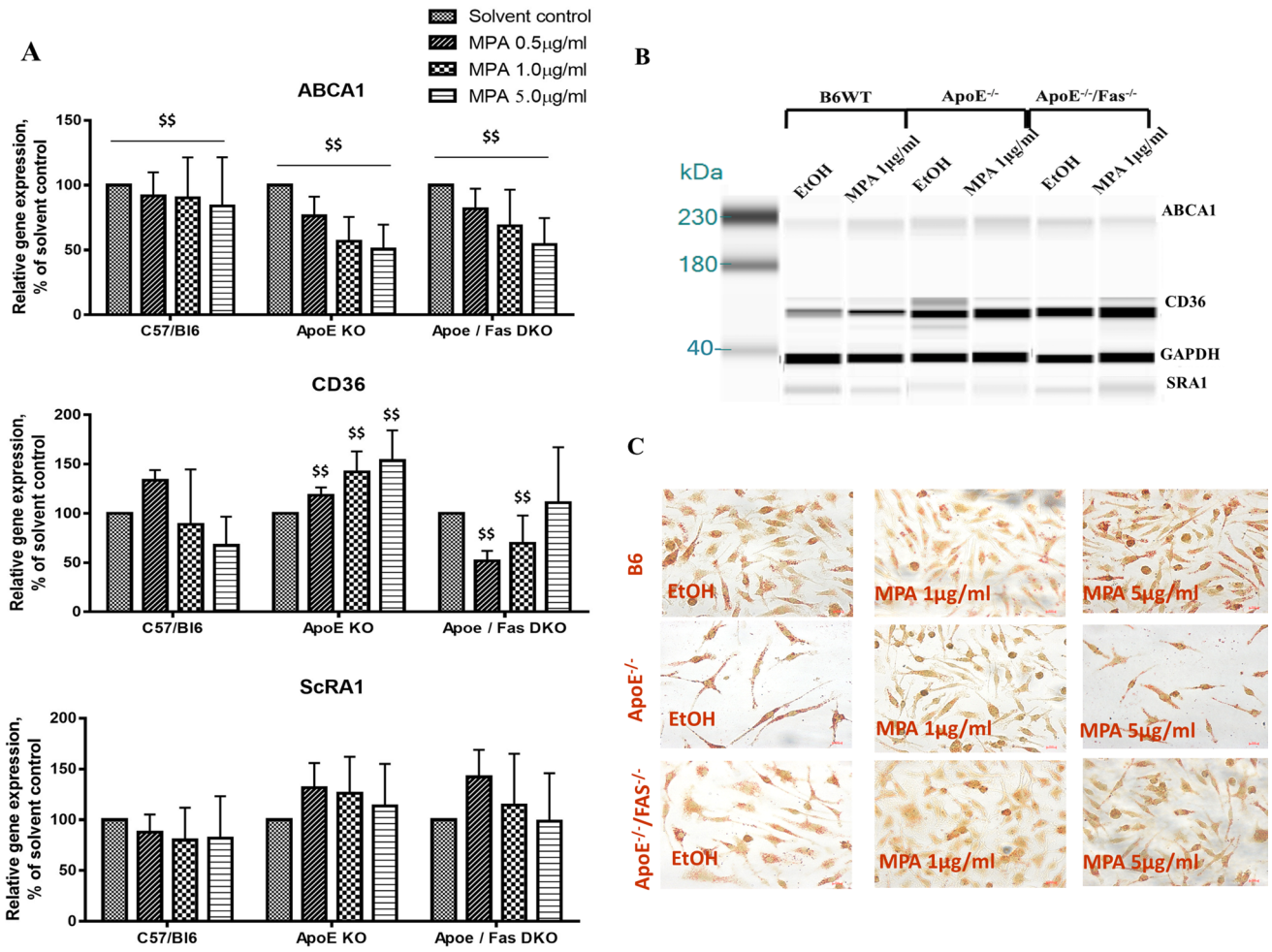

Fig. 4 Effect of MPA on the expression of key proteins involved in lipid transport in murine BMDM obtained from WT, ApoE$^{-/-}$ single and ApoE$^{-/-}$Fas$^{-/-}$ double knockout mice. BMDM were subjected to the same concentrations of MPA as human THP-1 macrophages, as indicated in the "Methods" section. Gene expression levels were calculated and graphed as change vs. solvent control set at 100%. MPA stimulated a concentration-dependent decrease in the expression of ABCA1 transporter in BMDM from all analyzed genotypes of mice (A). No significant changes were detected in CD36 message in macrophages from WT and ApoE$^{-/-}$Fas$^{-/-}$ (lupus-phenotype autoimmune and atherosclerosis-prone) mice. However, in BMDM from atherosclerosis-prone ApoE$^{-/-}$ mice, MPA stimulated a concentration-dependent increase in the expression of scavenger receptor CD36 vs. solvent control set at 100%. MPA had no effect on mRNA level of SR-A1 in all evaluated murine BMDM (**a**). Gene expression analysis was confirmed by Western blot (**b**). Changes in foam cell formation were detected by oil-red-O staining in subpopulations of macrophages incubated with MPA and in the presence of oxLDL for 24 h. Representative micrographs were photographed at ×40 (**c**)

suggested to be anti-atherogenic due to its anti-inflammatory properties and its presence in regressing plaques.

A pristane-induced lupus mouse model supports the contribution of abnormal macrophage polarization with disproportionate M1 over M2 predominance in the pathogenesis of lupus and treatment of these mice with a liver X receptor (LXR) agonist restored not only balance of polarization, but also ABCA1 levels [35, 36]. The inflammation that accompanies autoimmune conditions triggers abnormal lipid accumulation in the artery wall [37]. Thus, LDL is modified by reactive oxygen species to oxidized LDL (oxLDL), whose pro-inflammatory properties contribute to leukocyte recruitment into the arterial wall and monocyte differentiation into macrophages. It is very well accepted that the occurrence of elevated oxLDL in macrophages, accompanied by insufficient cholesterol efflux are key events in the progression of atherosclerosis [38–40]. Lipid overload leads to the formation of foam cells and serves as a pathological basis of atherosclerotic plaque. Our group has developed an approach for analyzing pro-atherogenic changes in lipid handling in human macrophages, screening the set of genes involved in lipid transport into the cell (influx) and out of the cell (efflux) [41]. This set includes: cell surface scavenger receptor class A1 (ScR-A), class B (CD36), class E (LOX-1)

and receptor CXCL16. All these receptors are involved in acceptance of oxidized LDL into the macrophages [38–40]. ABCA-1 and 27-hydroxylase expressions reflect the efflux properties of macrophages [42, 43].

Immunosuppressive drugs are the cornerstone of treatment for patients with autoimmune diseases [44, 45]. CsA is a clinical practice choice that is supported by reports of renal remission and improvement in SLE disease activity in patients with resistant proliferative lupus nephritis [46–49]. Another treatment option is MPA, which has been shown to improve outcomes in SLE patients as well [4, 10] and the combination of both drugs has been reported effective in a small group of refractory lupus nephritis cases [49, 50].

High risk of atherosclerosis is a problem in autoimmune diseases that may be impacted by immunosuppressive drugs through their effects on lipid handling in macrophages and other cells of the arterial wall. Therapeutic levels of both CsA and MPA in the blood of patients after treatment have been established and we used these as the basis for our studies [51, 52].

Several publications identify MPA as anti-atherogenic agent [53, 54]. Xu et al. [7] reported that treatment with MPA activates the PPARγ–LXRα–ABCA1 signaling pathway in vitro. Other studies confirmed beneficial effect of MPA in rabbit and mouse models of atherosclerosis [9–11, 53]. However, clinical studies have brought controversial results [15, 16, 54].

Unlike MPA, CsA has been shown to promote atherosclerosis, mainly by inhibition of reverse cholesterol transport, in vitro [55] and in mouse models [13, 54, 56] but was beneficial in a rabbit model of high-fat diet and balloon injury atherosclerosis [12, 57]. In humans, CsA treatment was associated with a reduction of cardiovascular mortality in a group of patients with diabetes after renal transplant [12, 20], as well as in patients with rheumatoid arthritis and SLE [21, 47, 49, 50].

The current study was designed to compare the effect of these two drugs applied simultaneously in the same cell systems—THP-1 human macrophages. We have observed that both MPA and CsA had different impacts on the expression of cholesterol efflux/influx proteins depending upon macrophage subtype. This suggests different pathways altered by these drugs in macrophages, depending upon their phenotype.

Thus, application of MPA in naïve THP-1 macrophages led to upregulation of the message and protein level of the ABCA1 transporter in a concentration-dependent manner, reaching $183.31 \pm 22.91\%$ vs. solvent control ($n = 3$, $P < 0.01$) at 5 μg/ml (Fig. 1a, d). That confirms all previous reports on the ability of MPA to stimulate the PPARγ–LXRα–ABCA1 signaling pathway in vitro [7]. In contrast, MPA had no effect on the expression of ABCA1 in M1 and M2 subpopulations of macrophages. The expression of 27-hydroxylase was not affected by MPA in M0 macrophages, but caused a significant augmentation of its level in M1 and M2 sub-populations (Fig. 1b–d). These results might indicate the existence of different pathways triggered by MPA in "immuno-compromised" macrophages.

CsA did not induce changes in the levels of ABCA1 or 27-hydroxylase when M0 and M1 macrophages were used (Fig. 1a, b). Similar results regarding ABCA1 expression with similar concentrations of CsA were demonstrated previously in M0 macrophages by Jin et al. [58]. However, CsA produced a great elevation in both ABCA1 transporter and 27-hydroxylase expressions in M2 macrophages. Thus, application of CsA resulted in more than threefold increase of ABCA1 mRNA and more than twofold increase in the mRNA level of 27-hydroxylase, compared to solvent control (Fig. 1c, d).

Our finding that CsA either did not affect or upregulate 27-hydroxylase expression is interesting because it is known that CsA is a non-competitive inhibitor that binds to the 27-hydroxylase enzyme, reducing its activity [59]. It is also known that CsA similarly binds directly to ABCA1, reducing its functionality [60].

Similar to macrophage cholesterol efflux, the two drugs trigger different pathways in cellular lipid uptake. Thus, MPA in the cell culture of naïve M0 macrophages did not alter the expression of the scavenger receptors evaluated in our study (CD36, SR-A1 and LOX1), with the exception of CXCL16. CXCL16 mRNA was increased to $152.78 \pm 33.01\%$ of solvent control upon exposure of M0 macrophages to 5 μg/ml MPA ($n = 3$, $P < 0.05$). In contrast, CsA augmented exclusively expression of LOX1 [$120.15 \pm 4.80\%$ ($n = 3$, $P < 0.05$)], but not other evaluated scavenger receptors (Fig. 2a, d). Yet again, analogous results were described earlier by Jin et al. [58] in THP-1 naïve macrophages.

In the M1 subpopulation of macrophages, MPA and CsA targeted the expression of the main scavenger receptors (CD36 and SR-A1) involved in lipid accumulation. However, they had an opposite effect on the receptors. Accordingly, the presence of MPA led to an increase in the CD36 mRNA message to $194.88 \pm 8.12\%$ at 0.5 μg/ml, to $181.66 \pm 12.45\%$ at 1 μg/ml and to $241.52 \pm 31.77\%$ at 5 μg/ml vs. solvent control set at 100% ($n = 3$, $P < 0.01$). On the contrary, exposure of M1 macrophages to CsA significantly downregulated expression of the CD36 ($n = 3$, $P < 0.01$) (Fig. 2b, d). Changes in the level of SR-A1 showed the same pattern MPA caused an increase in SR-A1 mRNA to $207.63 \pm 32.37\%$ at 0.5 μg/ml, to $150.69 \pm 14.13\%$ at 1 μg/ml and to $188.72 \pm 24.48\%$ at 5 μg/ml ($n = 3$, $P < 0.01$). Incubation with CsA had the opposite effect and led to a decrease in the expression of this scavenger receptor to $79.48 \pm 8.24\%$ vs. solvent control ($n = 3$, $P < 0.05$). The levels of LOX1 and

CXCL16 mRNA were not affected by the presence of either MPA or CsA in M1 phenotype macrophages (Fig. 2b, d).

An opposite effect on the expression of scavenger receptors was again detected in M2 macrophages upon exposure to MPA and CsA. Hence, MPA caused downregulation of SR-A1 and LOX1 message (Fig. 2c, d). SR-A1 message was reduced to $69.87 \pm 16.47\%$ by 0.5 μg/ml MPA ($n = 3$, $P < 0.05$). It remained suppressed to a similar level when cells were exposed to higher concentrations of MPA. In contrast, CsA caused a significant elevation in the expression of all scavenger receptors evaluated in this study (Fig. 2c, d).

We detected no significant changes in LDL uptake in the presence of MPA or CsA in all of the evaluated subtypes of macrophages (Fig. 3). Such results could be explained by compensatory triggering of counterbalancing changes in cholesterol efflux proteins when the drug triggered influx protein over-expression and vice verse. This type of compensation has been seen previously [61].

An intriguing observation involves the level of DiI-oxLDL internalization in M1 macrophages. We detected a spike in the DiI-oxLDL uptake in the presence of the lower dose of MPA (1 μg/ml) (Fig. 3b, d). This could be explained by a significant increase in the expression of scavenger receptors CD36 and SR-A1 at the same concentrations. However, this augmentation was not observed when the higher concentration was applied (5 μg/ml) (Fig. 3b, d). Taking into account that at the same time we identified upregulation in the expression of 27-hydroxylase, we postulate that compensatory mechanisms started to take effect in macrophages, limiting the accumulation of lipids.

To confirm our data, we used the same set of experimental conditions in murine BMDM. C57BL/6 J (wild type), ApoE $^{-/-}$ (a common knockout mouse model of atherosclerosis) and ApoE$^{-/-}$Fas$^{-/-}$ (a double knockout mouse with atherosclerosis and lupus-like disease) [24] mice were utilized for BMDM preparation. Once again, we observed a different response of diverse phenotypes of macrophages to MPA (Fig. 4). Interestingly, patterns found in human macrophages were replicated in murine macrophages. The same spikes altering expression of scavenger receptors were detected, similar to what we documented in human macrophages, and difference in oxLDL uptake in response to low and high concentrations of MPA was observed in murine BMDM, especially from ApoE$^{-/-}$ mice (Fig. 4c).

Another remarkable observation was an effect of CsA in M2 macrophages: simultaneous stimulation of cholesterol efflux and influx. As a result, no changes in DiI-oxLDL internalization were detected.

There are several limitations to this study. Animal experiments confirming the significance in vivo are planned as a next step. We did not evaluate detailed mechanisms by which CsA or MPA alter lipid handling. However, our results clearly underscore the importance of increased caution when using immunosuppressives in persons who are vulnerable to atherosclerosis due to autoimmune disorders.

Acknowledgements We thank Janet and Robert Buescher for their generous support.

Funding This work was supported by NIH/NCCAM Grant R21AT007032-01A1 and by the Elizabeth Daniell Research Fund.

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