Macrophage Apolipoprotein E and Proliferation of MCF-7 Breast Cancer Cells: Role of LXR

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Abstract. *Background: Apolipoprotein E (APOE), a lipid transport protein that has a key role in the lipoprotein metabolism, is expressed by macrophages under the control of the transcription factor Liver X Receptor (LXR), an oxysterol-activated transcriptional factor involved in cholesterol metabolism. Recent work has shown that LXR agonists may inhibit breast cancer cell proliferation in vitro. We hypothesized that LXR-activated macrophages, and in particular secreted macrophagic APOE, may potentiate the effect of LXR agonists. Our goal was to evaluate the effect of APOE, secreted by THP-1 macrophages under the control of LXR, on MCF-7 cell proliferation, a model of breast cancer. Materials and Methods: MCF-7 cells were incubated with supernatants from THP-1 cells previously treated with LXR agonists [T0901317 or 22(R)-hydroxycholestrol], or supernatants from THP-1 cells transfected with siRNA against APOE mRNA. Results: Viability assays and cell death quantification showed that media from LXR-activated macrophages reduced cell proliferation and increased apoptosis of MCF-7 cells. Interestingly, the opposite effects were observed when MCF-7 cells wre treated with media from the siRNA APOE-mediated knock-down model. Conclusion: This study highlights the protective role of LXR-activated macrophages against breast cancer growth, and the implication of APOE protein in the anti-proliferative and pro-apoptotic effects observed.*

Apolipoprotein E (APOE) is a key regulatory protein in lipoprotein metabolism (1) and is known to be a target of nuclear receptor Liver X Receptor (LXR) (2), which is crucial for regulating cholesterol homeostasis. APOE plays a role in reverse cholesterol transport (3, 4) and can serve as an extracellular cholesterol acceptor (5). It has been reported by numerous studies that APOE is capable of inhibiting the growth of several tumor cell lines (6-12), including breast carcinoma cells.

Moreover, in the past few years, anti-proliferative effects of synthetic and natural LXR agonists have been observed in various types of human cancer *in vitro* and in xenograft models such as prostate (13-15), ovarian (16, 17), colon (18), and breast cancer (19, 20). These studies reported that LXR agonists suppress the proliferation of cancer cells through induction of G_1 cell-cycle arrest or by inhibition of the AKT survival pathway. Recently we reported that anti-proliferative effects of LXR agonists on breast cancer cells may be correlated with enhanced extracellular cholesterol efflux (21).

Macrophages are found in or around tumors. In addition to their function as professional antigen-presenting cells, the tumoricidal activity of macrophages has been also studied with considerable interest. It is believed that macrophages present in the microenvironment of tumor cells may modulate their tumor growth. Many studies have indeed developed the use of immune cells, including macrophages, in immunotherapy against cancer in order to improve a better targeting of cancer cells (22-24). However, there are no studies on the effect of macrophages activated for LXR machinery on cancer cells.

In this study, we aimed to evaluate the effect of LXR-activated macrophages on breast cancer cells, and in particular to focus on macrophagic APOE. Our hypothesis is that APOE may potentiate the effect of LXR agonists. Therefore, our goal was to evaluate the effect of APOE secreted by macrophages, under the control of LXR, on MCF-7 cell proliferation, a model of breast cancer.

For this purpose, we used the monocytic cell line THP-1 which is frequently used as a macrophage cell model. These cells were treated with synthetic (T0901317) and natural [22(*R*)-hydroxycholesterol; 22(*R*)-HC] LXR agonists and the

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Table I. *Primer sequences used in this study.*

Gene	Gene Name	Forward sequence	Reverse sequence
ACTB	β-Actin	TGCTATCCAGGCTGTGCTATCC	GCCAGGTCCAGACGCAGG
BAX	BCL-2-associated X protein	ACCGTGACCATCTTTGTG	AAAACACAGTCCAAGGCA
BCL-2	B-cell lymphoma-2	AGGAGCTCTTCAGGGACGG	CGGACTCCACACACATGACC
APOE	Apolipoprotein E	CTGCGTTGCTGGTCACATTCC	CGCTCTGCCACTCGGTCTG

resulting macrophage-conditioned media were incubated with MCF-7 breast cancer cells and tested for their effects on proliferation and apoptosis. Furthermore, the expression of APOE was inhibited by siRNA transfection in THP-1 cells, and the effects of medium from this macrophage model were also evaluated on proliferation and apoptosis of MCF-7 cells.

Materials and Methods

Materials. Human breast cancer MCF-7 cells and human monocytic THP-1 cells were from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), T0901317, 22(*R*)-hydroxycholesterol [22(*R*)-HC] and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). 7-Aminoactinomycin D (7-AAD) was obtained from BD Biosciences (San Jose, CA, USA). Lipofectamine® RNAiMAX Transfection Reagent and Silencer Select® siRNAs were purchased from Invitrogen Life Technologies (Cergy Pontoise, France). All other reagents were purchased from Sigma Aldrich.

Cell culture. MCF-7 and THP-1 cells were cultured at 37˚C in a humidified incubator with 5% CO_2 in DMEM and RPMI medium respectively, both supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin. THP-1 cells were plated at a density of 1×10^6 in a 6-well plate and allowed to differentiate into macrophage-like cells with 100 nM PMA for three days. Then the seeding medium was removed and cells were treated with LXR agonists (T0901317 at 20 μM or 22(*R*)-HC at 2 μg/ml) diluted in 0.1% fatty acid-free bovine serum albumin (BSA)-containing medium for 24 hours at 37˚C. The resulting 24-h "THP-1+LXR agonists" medium was centrifuged to remove cell debris, and stored at –80˚C for future use.

RNA interference. Transfection of siRNA into THP-1 cells was performed using commercially-prepared siRNA duplexes (Invitrogen Life Technologies). The sequences of siRNAs (siAPOE and siControl) were not supplied. Duplexed siRNAs were resuspended in RNase-free water at a final concentration of 4 μM. Three days prior to the transfection, THP-1 cells were plated at a density of 5×105 in a 6-well plate in order to reach 60-80% confluence on the day of transfection, and allowed to differentiate into macrophage-like cells with 100 nM PMA. Subsequently, the differentiation medium was removed and cells washed twice with PBS. For each well, 5 μl of siRNA and 10 μl of Lipofectamine RNAiMAX were diluted in 500 μl Opti-MEM medium (Invitrogen Life Technologies), and the mix was incubated for 20-30 min at room temperature to allow the formation of siRNA/Lipofectamine RNAiMAX complex. The mix (Opti-MEM, siRNA and Lipofectamine) was then added directly onto the cells and the volume was set to 2 mL with RPMI medium/1% glutamine (without antibiotics), giving a final concentration of 10 nM for both siRNAs (siAPOE and siControl). Following an overnight incubation (~14 h) at 37˚C (5% CO_2 atmosphere), cells were washed twice with PBS and then incubated for an additional 24 h with fresh RPMI medium. The resulting 24 h "THP-1+siRNA" media were centrifuged to remove cell debris, and stored at –80˚C for future use.

APOE enzyme-linked immunosorbent assay. APOE secreted into macrophage-conditioned media ("THP-1+LXR agonists" or "THP-1+siRNA") was measured by ELISA using the Human Apolipoprotein E ELISA kit (Mabtech, Nacka Strand, Sweden) according to the manufacturer's instructions. Total cellular protein levels were determined using the BCA Protein Assay method (Sigma Aldrich).

Cell viability test. MCF-7 cells were plated at a density of 10^4 cells in 200 μl of culture medium per well in a 96-well plate and allowed to adhere overnight. Then the seeding medium was removed and cells were treated with 200 μl THP-1-conditioned media or with exogenous APOE protein (Abcam, Cambridge, UK) diluted at 20 μg/ml in 0.1% BSA-containing medium for 24 h or 48 h. For the MTT assay, 100 μl of medium were removed and 50 μl MTT (at 2.5 mg/ml) was added to each well at a final concentration of 833 μg/ml. The mixture was further incubated for 4 h, and the liquid in the wells was removed thereafter. Dimethyl sulfoxide (DMSO; 200 μl) was then added to each well to solubilize the formazan product and the absorbance was read at 570 nm. The relative cell viability was expressed as a percentage of the control cells.

Flow cytometric quantification of cell death. MCF-7 cells were treated with THP-1-conditioned media or exogenous APOE protein for 24 h in a 96-well plate. Cells were harvested with trypsin-EDTA (Sigma Aldrich) and homogenized. Cell death was estimated after DNA incorporation of fluorescent 7-AAD and fluorescence-activated cell sorting analysis using a BD Accuri® C6 flow cytometer (BD Biosciences). In this assay, cells with permeabilized plasma membrane stain with 7-AAD. Data are presented as the percentage of 7-AAD-positive (7-AAD+) cells.

RNA extraction and real-time quantitative-Polymerase Chain Reaction (qPCR). MCF-7 cells were plated at a density of 5×10^5 in a 6-well plate in 2 ml of culture medium and allowed to adhere overnight. Then the seeding medium was removed and cells were treated with THP-1-conditioned media for 24 h at 37˚C. THP-1 cells were collected after siRNA transfection or after treatment with LXR

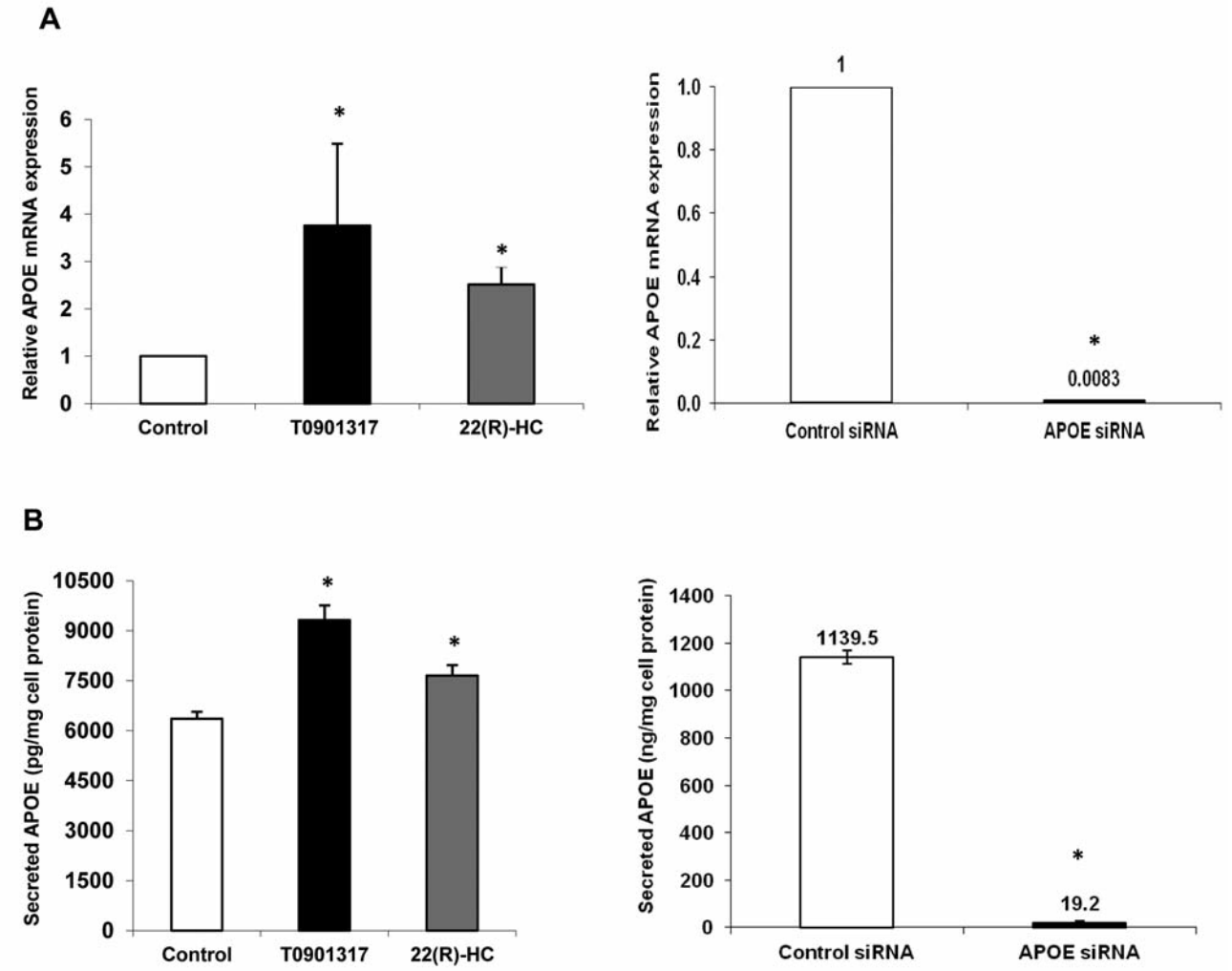

Figure 1. *Apolipoprotein E (APOE) inhibition in THP-1 cells after siRNA transfection. Differentiated THP-1 cells were treated with Liver X Receptor (LXR) agonists (T0901317 at 20 μM, or 22(R)-hydroxycholesterol [22(R)-HC] at 2 μg/ml) in serum-free medium for 24 h, or transfected with APOE siRNA or control siRNA at 10 nM using Lipofectamine® RNAiMAX. Cells were then harvested for mRNA extraction and supernatants from each model were collected and centrifuged (see the Materials and Methods section). A: Quantitative Polymerase Chain Reaction analysis for APOE gene expression levels. B: Secreted APOE levels in THP-1 supernatants evaluated by enzyme-linked immunosorbent assay. Data are the mean±SD of three different experiments. *p<0.05 versus untreated cells or control siRNA treatment using Student's t-test.*

agonists (see previous sections). For both cell lines, total RNA was isolated by the TriZol Reagent (Invitrogen) following the manufacturer's instructions. The mRNA (1 μg) was then reverse-transcribed into cDNA using Super-ScriptIII Reverse Transcriptase (Invitrogen). An initial denaturation step for 5 min at 70˚C was followed by an elongation phase of 45 min at 50˚C. qPCR was performed on a MyiQ2 Real-Time PCR Detection System (Bio-Rad, Marnes-la-coquette, France) using SYBR Green Supermix. The PCR was carried out for 45 cycles of 95˚C for 30 s and 60˚C for 30 s. The fluorescence was read during the reaction, allowing for continuous monitoring of the amount of PCR product. The values were normalized using β-actin as an endogenous internal standard. Relative quantification was performed using the $\Delta\Delta C_T$ method. The sequences of primers are shown in Table I.

Data analysis. Experiments were performed in triplicate and values shown correspond to the mean from at least three independent experiments. Student's *t*-test was used to comparisons, and *p*-values of <0.05 were considered as being significant.

Results

APOE expression in THP-1 cells after LXR activation and siRNA transfection. APOE is known to be stimulated in macrophages after LXR activation. Thus, we first wanted to test the effect of synthetic (T0901317) and natural (22(*R*)-HC) LXR agonists on *APOE* gene expression and secretion in the macrophage THP-1 cell line. As shown in Figure 1A,

qPCR results showed that treatment of THP-1 cells with T0901317 and 22(*R*)-HC for 24 h up-regulated *APOE* mRNA expression by 3.7- and 2.3-fold respectively ($p<0.05$). Furthermore, APOE secretion in THP-1 supernatants increased significantly ($p<0.05$) after LXR activation as shown in the ELISA assay (Figure 1B). In order to study the effect of macrophagic APOE on MCF-7 breast cancer cells, we generated THP-1 supernatants with reduced amounts of APOE. To do so, THP-1 cells were transfected with siRNA against *APOE* as detailed in the Materials and Methods section, and the inhibition of APOE was verified by qPCR and ELISA. Results showed that *APOE* mRNA levels, as well as secreted APOE protein were strongly inhibited after siRNA transfection (Figure 1). These results suggest that siRNA transfection was efficient since the inhibition was clear even after removing the transfection medium and replacing it with fresh RPMI medium for 24 h (see Materials and Methods).

Effect of THP-1-conditioned media on proliferation and apoptosis of MCF-7 cells. The poor in APOE medium ("THP-1+siRNA") was incubated with MCF-7 cells and compared with APOE-rich medium ("THP-1+LXR agonists"). Indeed, MTT assays showed that MCF-7 cell viability was reduced by 30% after a 24-h incubation with "THP-1+LXR agonists" medium compared to control cells (Figure 2A), while the "THP-1+siRNA" medium increased the viability by 40% after 24 h, and had almost no effect after a 48-h incubation (Figure 2B). Interestingly, MCF-7 cell viability also decreased by 18% and 40% after 24-h and 48-h incubation with exogenous APOE protein (Figure 2C).

The effect of THP-1-conditioned media on the expression of pro- and anti-apoptotic genes in MCF-7 cells was also evaluated by real-time qPCR. The results obtained showed up-regulation of the pro-apoptotic gene BCL-2-associated X protein (*BAX*) and a strong inhibition of the anti-apoptotic gene B-cell lymphoma 2 (*BCL2*) in MCF-7 cells treated with "THP-1+LXR agonists" medium compared to control medium (Figure 3A). On the other hand, treatment with "THP-1+siRNA APOE" medium reduced *BAX* expression compared to siRNA control medium, while no changes were observed for *BCL2* expression (Figure 3B).

Then we quantified cell death by flow cytometric analysis after 7-AAD staining. 7-AAD is a cell non-permeable DNA intercalating dye used for dead cell discrimination. Treatment of MCF-7 cells with "THP-1+LXR agonists" medium for 24 h increased the percentage of 7-AAD$^+$ cells compared to the control medium (Figure 4A), whereas this percentage decreased with "THP-1+siRNA APOE" medium compared to siRNA control medium (Figure 4B). Furthermore, treatment with exogenous APOE protein for 24 h resulted in a remarkable increase of 7-AAD$^+$ cells from 10.8% in untreated cells to 24.6% in treated cells.

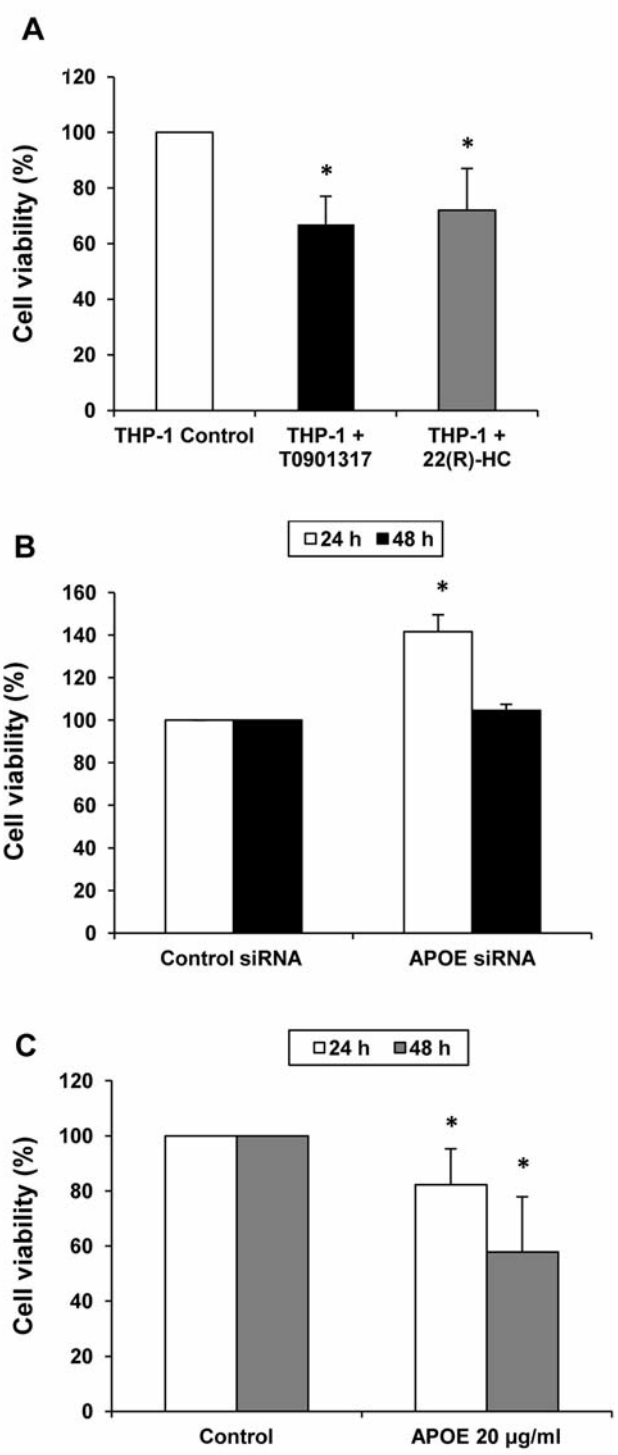

Figure 2. *Effect of THP-1-conditioned media on proliferation of MCF-7 cells. MCF-7 cells were treated with "THP-1+LXR agonists" medium (A), with "THP-1+siRNA" medium (B), or with exogenous APOE protein (20 µg/ml) diluted in serum-free medium (C). Cell viability was determined at 24 h or 48 h using the MTT assay. Data are the mean±SD of three different experiments. *p<0.05 versus control medium-treated cells (A), versus untreated cells (B), or versus control siRNA medium-treated cells (C) using Student's t-test.*

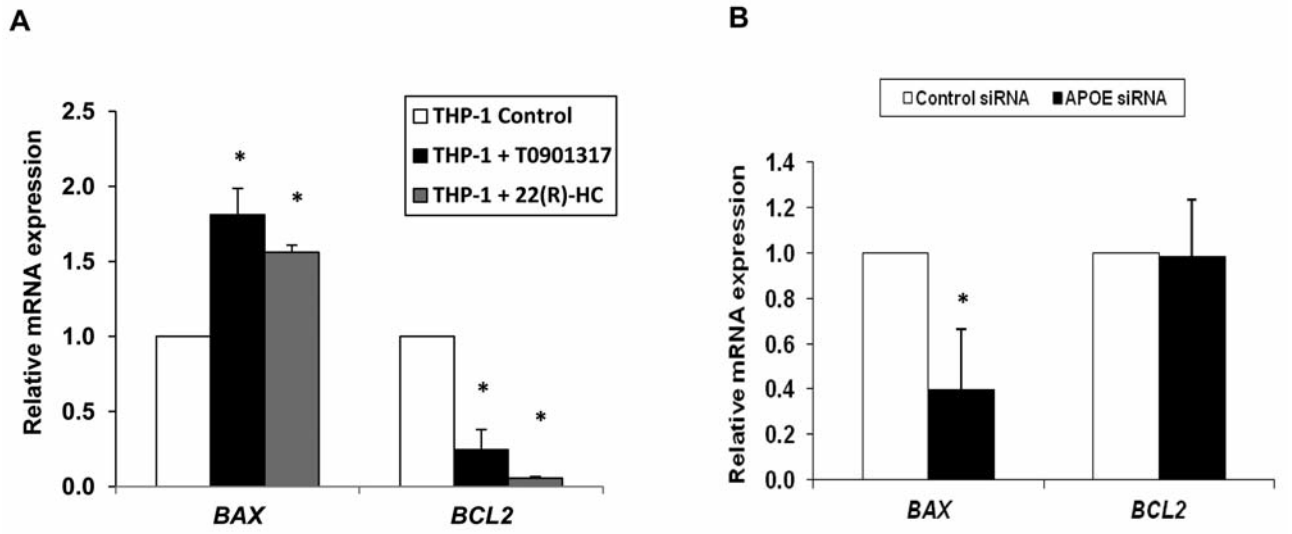

Figure 3. *Effect of THP-1-conditioned media on BAX and BCL2 genes in MCF-7 cells. MCF-7 cells were treated with "THP-1+LXR agonists" medium (A), or with "THP-1+siRNA" medium (B) for 24 h, and then harvested for mRNA extraction as detailed in the Materials and Methods section. BAX and BCL2 gene expressions were analyzed by quantitative Polymerase Chain Reaction. Data are the mean±SD of three different experiments each performed in triplicate. *p<0.05 vs. control medium-treated cells (A), or vs. control siRNA medium-treated cells (B) using Student's t-test.*

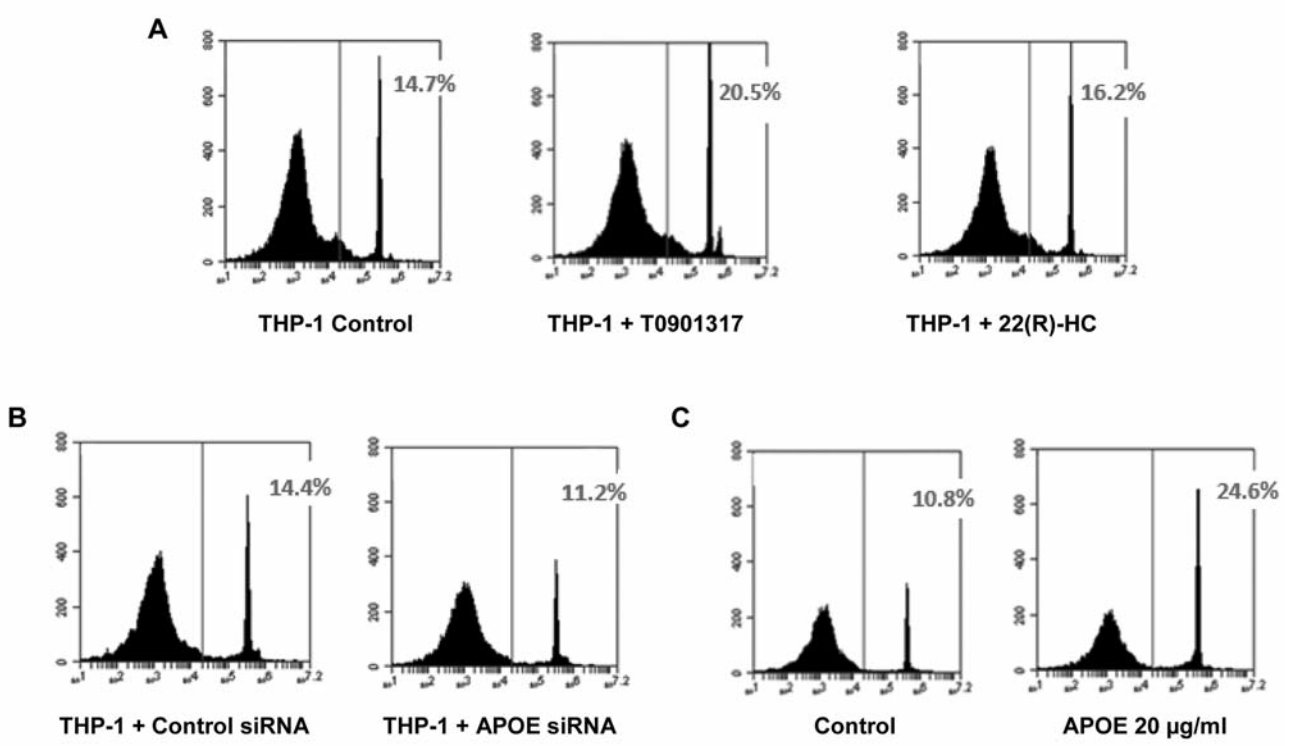

Figure 4. *Effect of THP-1-conditioned media on apoptosis of MCF-7 cells. MCF-7 cells were treated with "THP-1+LXR agonists" medium (A), with "THP-1+siRNA" medium (B), or with exogenous APOE protein (20 µg/ml) diluted in serum-free medium (C) for 24 h. Cell death was determined by flow cytometric analysis after 7-Aminoactinomycin D (7-AAD) staining. Data are presented as a percentage of the 7-AAD$^+$ cells. Results are representative of three independent experiments.*

Discussion

In this study we have shown for the first time the protective role of LXR-activated macrophages on MCF-7 breast cancer cells. Our results using macrophage-conditioned media from the THP-1 cell line treated with LXR agonists showed a powerful inhibitory effect on MCF-7 cell proliferation. Our data also showed an induction of apoptosis characterized by an overexpression of the pro-apoptotic gene *BAX* and a potent inhibition of the survival gene *BCL2*. Interestingly, results from siRNA-mediated knock-down of *APOE* mRNA in THP-1 cells pointed to a potential role of this protein in the anti-proliferative effect mediated by LXR-activated macrophages.

Our group, as well as others, have previously shown that LXR agonists have inhibitory effects on breast cancer growth (19, 20). Our previous findings highlighted the relationship between anti-proliferative effects of LXR and enhanced cholesterol efflux in MCF-7 cells *via* ATP-binding cassette G1 (ABCG1) cholesterol transporter (21).

Secretion of APOE by LXR-activated macrophages is thought to contribute to cholesterol efflux and thus to regulate macrophage membrane cholesterol concentration (25). However, no studies were conducted to verify if macrophagic APOE secretion is associated with anti-proliferative effects on cancer cells. Expression of APOE is believed to be low or absent in breast cancer tissues and breast cancer cell lines, including MCF-7 (9). It has been reported by numerous studies that APOE is capable of inhibiting the growth of several tumor cell lines (6-12), while intriguingly, other studies showed that APOE is required for the proliferation and survival of other cancer types, such us ovarian cancer (26) and lung adenocarcinoma (27). Therefore, it is believed that this duality of APOE activity on cancer cells is tissue-specific, and its relationship with some cancer types is still under debate. Moreover, the status of APOE protein, associated or not with lipids in lipoprotein particles, could be a key mediator for its effect on cancer cell proliferation. The studies mentioned above did not provide clear evidence to resolve this question, since they used residues of peptides derived from the APOE protein (8, 10) or transfected *APOE* in plasmid vector into cancer cells (9).

This study revealed the anti-proliferative and pro-apoptotic effects of LXR-activated macrophages on MCF-7 breast cancer cells. The siRNA-mediated knock-down of macrophagic *APOE* demonstrated that this protein is indeed involved in the effects observed by using macrophage-conditioned media. We suggest that macrophagic APOE may potentiate the effect of LXR agonists, without eliminating the hypothesis that other mediators of the LXR machinery could also be implicated. More investigations are needed in order to clarify the mechanism by which macrophagic APOE acts to block breast cancer growth. Then potential targeting of LXR in immunotherapy using macrophages could be planned.

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