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Original Research

Obesity-Derived Biomolecules Promote the Differentiation of THP1 Monocytes to Macrophages *In Vitro*

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Abstract

Background: It is well known that the microenvironment in which an immune response develops, generally pro-inflammatory or immunosuppressive, along with other overproduced biomolecules recognized by pattern recognition receptors, may promote the stimulation and differentiation of monocytes into macrophages with effector functions. Low-density lipoprotein (LDL) plays a fundamental role in cholesterol transport. By contrast, its oxidized form (ox-LDL), which is overexpressed in conditions of obesity and chronic low-grade inflammation, has been associated with cardiovascular diseases. Depending on the microenvironmental context, prostaglandin E2 (PGE2) participates in various scenarios such as inflammation, anti-inflammation, and homeostasis. Therefore, obesity-derived biomolecules such as LDL, ox-LDL, and PGE2 could induce the differentiation of immune cells into effector populations with either pro-inflammatory or immunosuppressive profiles. **Methods**: In the present work, we studied the effects of LDL, ox-LDL, and PGE2 on the differentiation of the human THP1 monocytic cell line into macrophages under two different protocols, analyzing several activation markers associated with either pro-inflammatory M1 or anti-inflammatory M2 profiles by flow cytometry and quantitative PCR (qPCR). **Results**: Our data suggest that native LDL induces the differentiation of human THP1 monocytes into M1 macrophages even more efficiently than classic phorbol 12-myristate 13-acetate (PMA) stimulation, whereas ox-LDL and PGE2 induce the expression of activation markers similarly to interferon gamma or interleukin 4 during PMA preactivation of macrophages. **Conclusions**: The results of this study add evidence to the role of obesity-derived biomolecules as non-canonical differentiation stimuli in macrophages, which could be relevant in contexts where these biomolecules are chronically overproduced, such as obesity, low-grade inflammation, type 2 diabetes, and cancer.

Keywords: macrophages; monocytes; immune response; activation profile; low-density lipoprotein (LDL); oxidized LDL; prostaglandin E2; flow cytometry

1. Introduction

Overweight and obesity are a public health problem with a prevalence of nearly one billion people worldwide [1,2]. During their development, both overweight and obesity induce a particular phenomenon called low-grade inflammation, causing chronic activation of the immune response, which in turn is associated with comorbidities such as hypertension, dyslipidemia, type 2 diabetes, and cancer [3,4]. In the microenvironment where the effector functions of immune cells are developed, such as low-grade inflammation induced by obesity, there are no homogeneous and unique stimuli that clearly polarize immune cell populations towards a specific profile. Instead, there is an interaction with a wide variety of obesity-related biomolecules that induce distinct and multiple phenotypes [5], affecting immune cells' effector capacity and activation profile. Oxidized low-density lipoprotein (ox-LDL) can differentiate monocytes into macrophages with a proinflammatory profile [6] in a macrophage colony-stimulating factor (M-CSF)-dependent manner [7]. This ox-LDL originates from the oxidation of LDL under conditions of constant stress, which is common in individuals with obesity or elevated triglycerides and cholesterol [8]. It can trigger cardiovascular diseases such as atherosclerosis [6]. Prostaglandin E2 (PGE2) is key in differentiating immune cells during chronic inflammatory processes. Although PGE2 levels increase in conditions such as atherosclerosis, PGE2 can also promote the polarization of macrophages towards an alternatively activated or M2 profile [9], which favors immunosuppression. Finally, macrophages exposed to native LDL can transform into foam cells storing cholesterol [10]; however, native LDL can induce the differentiation of peripheral blood monocytes into macrophages [11], although its M1/M2 profile is still undefined. Therefore,

although the literature has shown that classic individual stimuli such as lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) have effects on the activation and differentiation of monocytes and macrophages, it is feasible that direct stimulation of these myeloid cells with LPS, PMA, or obesity-derived biomolecules could induce a completely different effect if monocytes are first preactivated with PMA [12] and subsequently differentiated with obesity-derived biomolecules. This scenario, despite its limitations, would simulate the effects of *in vivo* processes, where immune cells are constantly stimulated by pathogen- or damage-associated molecular patterns, which are recognized by pattern recognition receptors [13].

In this work, we studied the effects of the biomolecules LDL, ox-LDL, and PGE2 on the differentiation of the THP1 human monocytic cell line into cluster of differentiation 68-positive ($CD68^+$) macrophages *in vitro*, evaluating the activation markers CD14, CD16, Toll-like receptor 2 (TLR2), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) programmed death ligand-2 (PD-L2), and galectin 9 (Gal9) by flow cytometry. We also described and compared these effects to those of classical LPS, PMA, interferon gamma (IFN-γ), and interleukin 4 (IL-4) stimulation. We used two different stimulation protocols: individual contact for 72 h with obesity-derived molecules, and prestimulation for 48 h with PMA followed by treatment for 72 h with obesity-derived molecules. We analyzed the relative expression of IL-1β and nitric oxide synthase 2 (NOS2) by quantitative PCR (qPCR). We found that stimulation with native LDL for 72 h promoted the differentiation of THP1 monocytes into $CD68^+$ macrophages even more efficiently than PMA stimulation, and these cells exhibited a dual M1 activation phenotype. Meanwhile, the pre-differentiation of THP1 monocytes with PMA for 48 h, followed by stimulation with LDL, ox-LDL, or PGE2 for 72 h, increased the expression of both M1 and M2 activation markers. Therefore, stimulation of monocytes with biomolecules overexpressed during obesity and obesity-related diseases promotes their differentiation into macrophages.

2. Materials and Methods

2.1 In Vitro Cell Culture

For this work, the THP1 human monocytic cell line (ATCC, Manassas, VA, USA) was cultured in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Mayimex Inc., Mexico City, Mexico) and 2-mercaptoethanol 0.05 mM (Sigma, St. Louis, MO, USA) at 37 °C with 5% CO_2. Culture density did not exceed 2×10^6 cells/mL. Cell concentration and viability were assessed by a 1:1 dilution with trypan blue using the Countess II FL Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA), always maintaining viability above 90% (data not shown). THP1 cell line was validated by STR analysis (intraspecies) and tested negative for mycoplasma contamination by Hoechst DNA Stain, agar culture method and PCR-based assay (ATCC).

2.2 Maintenance of the THP-1 Cell Line

A frozen vial of THP-1 cells was thawed in a 37 °C water bath for approximately 2 min. Once removed from the bath, the vial content was transferred to a 15 mL conical tube (Falcon, Corning, NY, USA) containing 9 mL RPMI complete medium, which was centrifuged at 600 rpm for 5 min at 25 °C. The pellet was decanted, and 10 mL saline solution was added for a second wash, followed by centrifugation under the same conditions. The pellet was resuspended in 5 mL complete culture medium and placed in a 25 cm^2 culture flask (Corning, Corning, NY, USA), which was incubated at 37 °C with 5% CO_2 in the dark until reaching a confluence of 1×10^6 cells/mL. When THP1 cells reached 80% confluence in the culture flask, total volume was recovered from the flasks into a 15 mL conical tube (Falcon) and centrifuged at 1500 rpm for 5 min at 37 °C, resuspending in 1 mL RPMI complete medium until the pellet was dissolved entirely. Next, 500 µL THP1 cells were added to 25 cm^2 culture flasks (Corning) with 4.5 mL complete medium. This procedure was performed every 2 days, three times a week. To freeze THP-1 cells, the total volume from the flasks (approximately 5×10^6 cells) was recovered into a 15 mL tube and centrifuged at 1500 rpm for 5 min at 37 °C. The supernatant was discarded, and the pellet was resuspended in RPMI medium supplemented with 20% FBS and 5% DMSO. The contents were transferred to a cryovial and placed immediately in an ultra-freezer (Haier Biomedical, Wilmington, DE, USA) at –70 °C until further use.

2.3 Differentiation of THP-1 Monocytes

A total of 200,000 monocytes were cultured in non-adherent 24-well culture plates (Corning) in triplicate for 24, 48, and 72 h. The negative control consisted of unstimulated THP1 monocytes. Cells were stimulated with 250 ng/mL LPS (Sigma), 50 ng/mL PMA (Sigma), and 250 ng/mL LPS plus 50 ng/mL PMA. THP-1 cells were stimulated for 72 h with 100 µg/mL LDL (Invitrogen, Carlsbad, CA, USA), 100 µg/mL ox-LDL (Invitrogen), and 10 ng/mL PGE2 (Sigma). In the differentiation experiments with IFN-γ and IL-4, 20 ng/mL of each cytokine (PeproTech/Thermo Fisher Scientific) was added. In the monocyte to macrophage pre-differentiation experiments, THP-1 monocytes were stimulated as previously described [12]. After 48 h of stimulation with 200 ng/mL PMA, the cells were washed with fresh RPMI medium, initiating a crucial 24-h rest period. According to the literature, these PMA-pretreated cells were then exposed to the corresponding stimuli for the next 72 h, ensuring a comprehensive differentiation process.

2.4 Flow Cytometry

For phenotype characterization of THP1 monocytes differentiated into macrophages, cells were collected after the respective stimulation protocol into 5 mL round-bottom tubes (Sarstedt Group, Newton, NC, USA). A double wash was performed in each well of culture plates with 200 µL FACS sheath fluid (BD Biosciences, Franklin Lakes, NJ, USA), centrifuging at 1500 rpm at room temperature for 5 min. The supernatant was decanted, and the pellet was resuspended in 50 µL FACS sheath fluid with antibodies targeting the surface molecules to be analyzed by flow cytometry. The antibodies were titrated to evaluate the optimal concentration for every sample. The antibodies used were anti-CD16-PE (50-0166-T100) and anti-CD14 Violet-Fluor 450 (75-0149-T100) (Cytek Biosciences, Fremont, CA, USA), anti-PD-L2 APC/Cy7 (345516), anti-LOX-1-APC (358605), anti-TLR2-PE/Cy7 (309722), anti-Gal9-FITC (348911), and anti-CD68-APC/Cy7 (333822) (Biolegend, San Diego, CA, USA). Once the antibodies were added, the sample was mixed using a vortex and incubated in the dark at 4 °C for 30 min. To stop the reaction, 1 mL FACS sheath fluid was added, and the sample was centrifuged at 1500 rpm for 5 min at 4 °C. Finally, the supernatant was decanted, the pellet was resuspended in 300 µL FACS sheath fluid, and samples were analyzed using the Attune NxT Flow Cytometer (Thermo Fisher Scientific). Twenty thousand events were recorded from the monocyte cell population defined by size, measured by forward scatter area (FSC-A) and granularity, measured by side scatter area (SSC-A).

2.5 Sorting

Two million THP1 cells were added to 25 cm^2 culture flasks (Corning) and stimulated with either PMA or LDL for 72 h. THP1 cells were stained with anti-CD68-APC/Cy7 antibody (Biolegend) as described. Cells were selected by FSC-A, forward scatter height, and SSC-A characteristics and the $CD68^+$ cells with a purity greater than 95% were sorted in the FACSAria Fusion Flow Cytometer (BD Biosciences).

2.6 qPCR

Total RNA was extracted from sorted THP1 cells using Trizol® (Invitrogen). RNA concentration was determined by measuring the absorbance at 260 nm. One microgram of RNA was used for first-strand cDNA synthesis with the Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Gene expression was assessed with the 2X Universal SYBR Green Fast qPCR Mix (ABclonal, Woburn, MA, USA) in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Relative gene expression values were normalized to the constitutive expression of the RPLP0 gene encoding 60S acidic ribosomal protein P0. Values were determined using the $2^{-\Delta\Delta CT}$ method. The specific primers sequences were as follows: inducible NOS (*iNOS*) F: 5′-CCCTTCCGCAGTTTCTGGCAGCAAC-3′ R: 5′-ATGTCCTGAACATAGACCTTGG3′; *IL-1*β F: 5′-ATGATGGCTTATTACAGTGGCAA-3′ R: 5′-GTCGGAGATTCGTAGCTGGA-3′; tumor necrosis factor alpha (*TNF-*α) F: 5′-TCTCGAACCCCGAGTGACAA-3′ R: 5′-TACTCTCAGCTCCACACCA-3′; and *arginase* F: 5′-CTTGTTTCGGACTTGCTCGG-3′ R: 5′-CACTCTATGTATGGGGGCTTA-3′.

2.7 Statistical Analyses

Flow cytometry samples were analyzed using FlowJo V10 software (BD Biosciences), To evaluate the statistical differences between experimental groups, the Student's *t*-test was performed with the Shapiro–Wilk test resulting in W value >0.8 and p-value > 0.05, signifying that the data could be considered normally distributed (Gaussian distribution). For multiple comparisons, one-way analysis of variance (ANOVA) was used followed by the Dunnett's multiple comparison test with unstimulated cells as the control group, using Prism version 8.0.2 (GraphPad, La Jolla, CA, USA). We tested the normality and homoscedasticity in each graph according to one-way ANOVA in the GraphPad Prism program using the Bartlett's test. We always had $p > 0.05$ when assuming the data equality of variances. Each graph shows the mean and the standard error of the mean. All samples were obtained in duplicate.

3. Results

Monocytes stimulated with PMA or LPS can express both the CD14 molecule, which is one of the receptors that recognize LPS [14], and the CD16 molecule, which is the receptor that recognizes the heavy chain of immunoglobulin FcγRIII [15]. Therefore, our THP1 monocyte culture was stimulated with LPS, PMA, or a combination of LPS and PMA for 24, 48, or 72 h as described in the Materials and Methods (Fig. 1A). The percentage of $CD14^+CD16^+$ was evaluated by flow cytometry (Fig. 1B). We found that stimulation with PMA and the combination of PMA and LPS were more efficient at inducing the $CD14^+CD16^+$ monocyte population, which was time-dependent and reached the highest percentage after 72 h of stimulation (Fig. 1C,D).

Next, we evaluated whether obesity-associated molecules LDL, ox-LDL, and PGE2 may induce a higher percentage of activation markers in THP1 monocytes after 72 h of stimulation. Monocytes were defined by CD16 positivity, and the percentage of CD14, TLR2, LOX-1, PD-L2, and Gal9 markers was simultaneously evaluated based on the analysis strategy described (Fig. 2A). We observed that monocytes exposed to both LDL and ox-LDL displayed a higher percentage of the CD14 population within the $CD16^+$ population, even more efficiently than LPS plus PMA stimulation (Fig. 2B,C). We also found that the combination of LPS plus PMA stimulus on monocytes increased the percentage of the non-classical markers

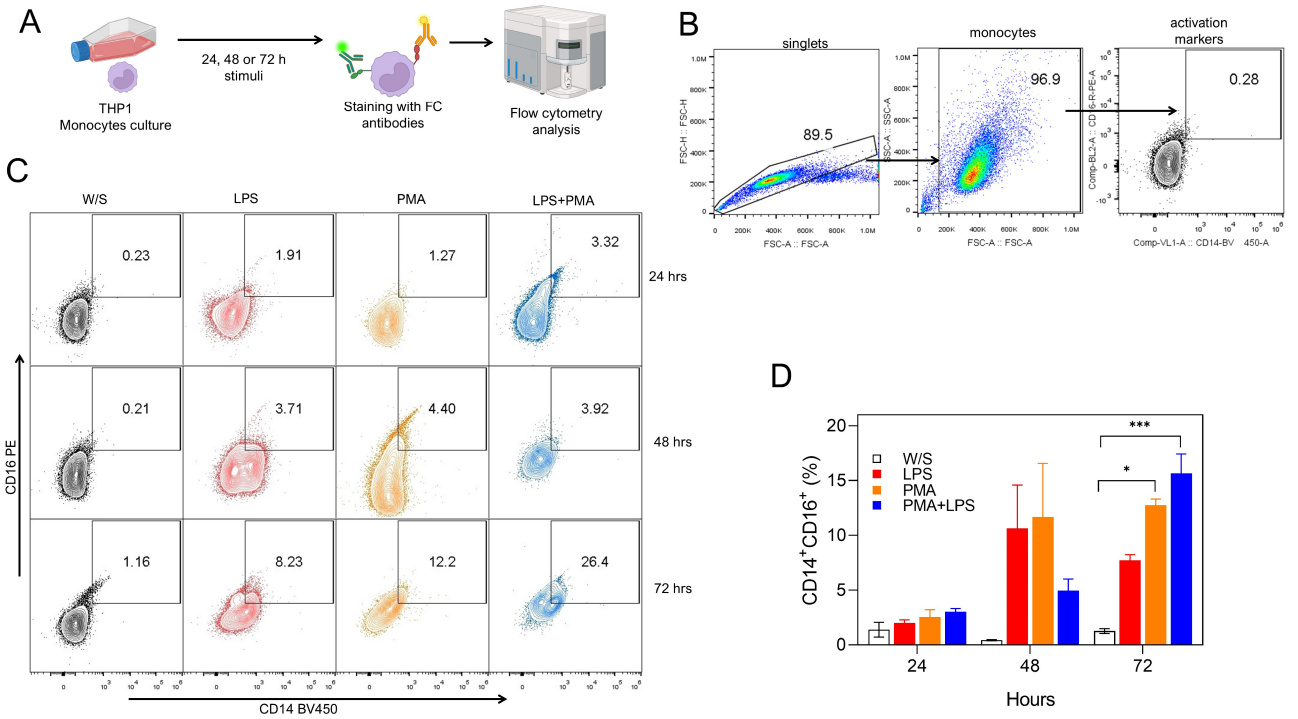

Fig. 1. Both PMA and LPS induced the activation of monocytes *in vitro*. (A) Experimental strategy. THP1 cells were cultured for 24, 48, or 72 h with LPS, PMA, or LPS+PMA, after which cells were stained with fluorochrome-coupled antibodies and analyzed by flow cytometry. (B) Analysis strategy for CD14 and CD16 percentage obtained by flow cytometry, where singlets were selected after stimulated monocytes were defined by their FSC-A and SSC-A characteristics. Finally, monocytes were selected by CD14 and CD16 coexpression. (C) Representative (left) and (D) total (right) data obtained from nine independent experiments conducted in duplicate for CD14 and CD16 percentages. W/S: without stimulus in all figures. Statistical differences were assessed by one-way ANOVA followed by the Dunnett's multiple comparison test with unstimulated cells serving as the control group. $*p = 0.0208$ and $***p = 0.0002$. and CD16 percentages. PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; FSC-A, forward scatter area; FSC-H, forward scatter height; SSC-A, side scatter area; ANOVA, analysis of variance; FC, flow cytometry.

LOX-1 and PD-L2, suggesting that monocytes under these stimuli showed a dual activation profile (Fig. 2B,C) since they simultaneously expressed both classical and alternative activation markers. On the other hand, monocytes stimulated with LDL and ox-LDL did not promote increasing percentages of the LOX-1 molecule, but a significant reduction of PD-L2 expression was observed within the $CD16^+$ population (Fig. 2B,C). The percentage of $Gal9^+$ cells within the CD16 gate increased after LDL exposure (Fig. 2B,C). Stimulation with PGE2 induced a slight, non-significant increase in most of the markers evaluated. Also, the $CD16^+$ monocyte population constitutively expressed the TLR2 receptor under all stimuli analyzed (Fig. 2B,C). These data suggest that the combination of PMA plus LPS stimulus induces the dual expression of classical and non-classical receptors in the THP1 monocyte population, whereas obesity-associated molecules such as LDL and ox-LDL favor a classical profile of $CD14^+CD16^+$ monocytes, preventing the expression of molecules associated with suppressive activities on the immune response.

In addition to studying the effect of obesity-associated molecules on the activation of THP1 monocytes, we also determined whether LDL, ox-LDL, and PGE2 could induce monocyte differentiation into $CD68^+$ macrophages after 72 h of stimulation. We simultaneously analyzed the percentage of $CD14^+$ and $CD16^+$ cells within the $CD68^+$ macrophage population (Fig. 3A). We evaluated the effect of classical stimuli, such as PMA or cytokines like IFN-γ and IL-4, on the differentiation of $CD68^+$ macrophages after 72 h of stimulation. We found that individual stimulation with IFN-γ and IL-4 for 72 h induced a slight increase in $CD68^+$ macrophages, with a low percentage of the $CD14^+$ and $CD16^+$ population (Fig. 3B,C). PMA significantly increased the percentage of $CD68^+$ macrophages but not $CD68^+CD14^+$ (Fig. 3B,C). However, the most significant effect was observed with individual stimulation by native LDL, where we detected a significant increase in the percentage of $CD68^+$, $CD68^+CD14^+$ and $CD68^+CD16^+$ cells even more efficiently than either classical cytokine or PMA stimulus (Fig. 3B,C). Ox-LDL and PGE2 did not induce the differentiation of monocytes into $CD68^+$

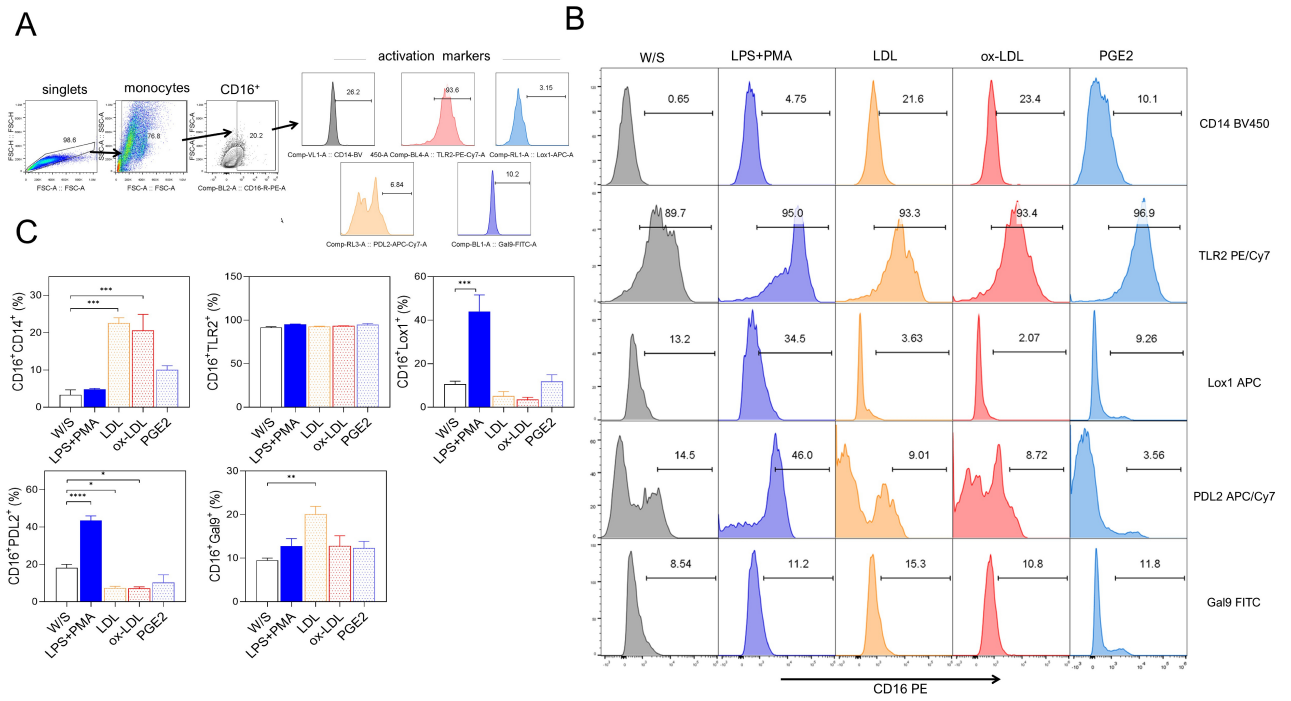

Fig. 2. Native LDL and ox-LDL induced a pro-inflammatory profile in THP1 monocytes. (A) THP1 cells were cultured for 72 h with LPS+PMA, LDL, ox-LDL, or PGE2, and analyzed for CD14, CD16, TLR2, LOX-1, PD-L2, and Gal9 percentages by flow cytometry according to the analysis strategy, where singlets were selected after stimulated monocytes were defined by their FSC-A and SSC-A characteristics to select $CD16^+$ cells expressing CD14, CD16, TLR2, LOX-1, PD-L2, and Gal9. (B) Representative and (C) Total data obtained from three independent experiments performed in duplicate. Statistical differences were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test with unstimulated cells as the control group. $***p = 0.0003$ for LDL and $***p = 0.0007$ for ox-LDL in $CD16^+CD14^+$ population; $***p = 0.0004$ in $CD16^+LOX\text{-}1^+$ population; $****p < 0.0001$ for LPS+PMA, $*p = 0.0116$ for LDL, and $*p = 0.0270$ for ox-LDL in $CD16^+PD\text{-}L2^+$ population; $**p = 0.0028$ in $CD16^+Gal9^+$ population. LDL, low-density lipoprotein; ox-LDL, oxidized low-density lipoprotein; PGE2, prostaglandin E2; TLR2, Toll-like receptor 2; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; PD-L2, programmed death ligand-2; Gal9, galectin 9.

macrophages, or $CD68^+CD14^+$ cells, only was observed a non-significant increment in $CD68^+CD16^+$ by ox-LDL stimulus. To further analyze the effect of PMA and LDL exposure on THP1 monocytes, we performed qPCR assays to assess *IL-1β*, *iNOS*, *arginase*, and *TNF-α* gene expression in sorted $CD68^+$ cells stimulated with either PMA or native LDL (Fig. 4A,B). After 72 h of stimulus, we observed that both PMA and LDL induced an increased percentage of $CD68^+$ cells (Fig. 4C). The purity of sorted cells was >95% and 98% with both PMA and LDL, respectively (Fig. 4C). We observed that native LDL stimulus induced the slight expression of M1 macrophages functional markers such as *IL-1β* and *iNOS* (Fig. 4D); however, arginase and TNF-α gene expression was unaffected (data not shown). Together, these data suggest that native LDL, after 72 h of stimulation, has the remarkable capacity to efficiently induce $CD68^+$ macrophages.

Since we found that native LDL promotes the differentiation of THP1 monocytes into macrophages, we evaluated whether the obesity-associated molecules LDL, ox-LDL, and PGE2 may induce changes after pre-differentiating monocytes into macrophages with PMA [12]. Based on this experimental strategy, THP1 monocytes were stimulated with PMA for 48 h and characterized by CD11b and CD68 positivity (Fig. 5B), showing that PMA stimulation significantly increased the percentage of $CD11b^+CD68^+$ macrophages (Fig. 5C). Subsequently, the cultured cells were rested for 24 h and then stimulated for 72 h with obesity-associated molecules, as well as classical stimuli such as PMA and cytokines IFN-γ and IL-4 (Fig. 5A). Under these conditions (48 h with PMA plus 72 h of stimulation), PMA induced a higher percentage of macrophages, whereas all other stimuli, including both cytokines and obesity-associated biomolecules, induced monocyte-to-macrophage differentiation more efficiently (Fig. 5E,F) than PMA-individual 72-h stimulation (Fig. 3B,C). Additionally, we analyzed the percentage of activation markers within $CD68^+$ macrophages described in Fig. 5D. We observed that LDL continued to promote macrophage differentiation with a classical $CD68^+CD16^+$ profile. Still, un-

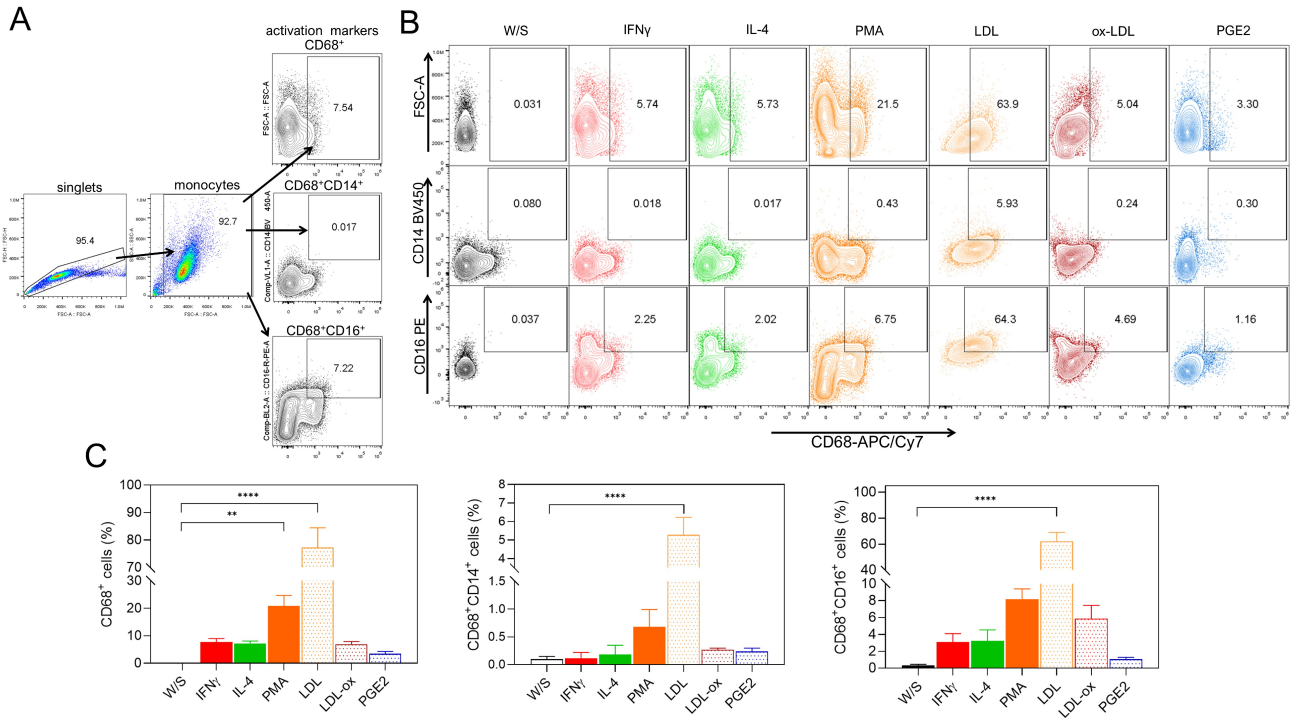

Fig. 3. Native LDL and ox-LDL induce the differentiation of THP1 monocytes to CD68$^+$ macrophages. (A) THP1 cells were cultured for 72 h with IFN-γ, IL-4, PMA, LDL, ox-LDL, or PGE2, and analyses for CD68, CD14, and CD16 percentages were performed by flow cytometry according to the analysis strategy, where singlets were selected after stimulated monocytes were defined by their FSC-A SSC-A characteristics to select CD68$^+$, CD68$^+$CD14$^+$, or CD68$^+$CD16$^+$ cells. (B) Representative and (C) Total data obtained from four independent experiments performed in duplicate. Statistical differences were performed by one-way ANOVA followed by Dunnett's multiple comparison test with unstimulated cells serving as the control group. $^{**}p = 0.0018$ in CD68$^+$ population for PMA and $^{****}p < 0.0001$ in CD68$^+$ population for LDL; $^{****}p < 0.0001$ in CD68$^+$CD14$^+$ population for LDL; $^{****}p < 0.0001$ in CD68$^+$CD16$^+$ population for LDL. CD68$^+$, cluster of differentiation 68-positive; IFN-γ, interferon gamma; IL-4, interleukin 4.

der these stimulation conditions, native LDL also allowed the induction of CD68$^+$CD14$^+$ macrophages (Fig. 6A,B). Unlike direct monocyte stimulation, only PMA stimulation favored an increase in the percentage of Gal9, and there was no significant difference in the expression of LOX-1 in CD68$^+$ macrophages under all stimuli (Fig. 6A,B). However, macrophage pre-activation and subsequent stimulation with cytokines or obesity-associated molecules favored the increased expression of CD68$^+$PDL2$^+$ macrophages, and such an effect was more pronounced with PMA and native LDL stimulation (Fig. 6A,B). There was no synergistic effect when macrophages were stimulated with native LDL and PGE2 (Fig. 6A,B). All of these data suggest that macrophage pre-activation with PMA followed by stimulation with cytokines, PMA, or obesity-associated biomolecules favors the expression of a dual profile in macrophages derived from THP1 monocytes.

4. Discussion

Monocytes and macrophages are myeloid cells with essential mechanisms for immunity generation; monocytes develop their functions mainly in blood, macrophages are differentiated in tissues where they are involved in processes like phagocytosis, antigen presentation, cytokine production, or tissue repair [16]. Classical, intermediate, and non-classical nomenclature has been described for blood monocytes based on CD14 and CD16 expression [17]. However, this classification is not homogeneous in THP1 cells, where it has been widely accepted that either PMA [18,19] or LPS stimulation induces a pro-inflammatory profile [20–22]. Under controlled *in vitro* conditions, the classical or M1 macrophage profile is obtained from monocytes stimulated with either PMA [20], LPS [23], cytokines such as IFN-γ [24], or M-CSF [25]. The alternative macrophage profile, or M2, is obtained through either stimulation with cytokines such as IL-4, IL-13, or IL-10 [26] or helminth-derived products [27]. However, some studies including *in vitro* and *ex vivo* [28] and single-cell RNA sequencing, which combines the analytical characteristics of flow cytometry and massive sequencing, have described that the macrophage profile is not homogeneous and a variety of genes with diverse functions can be expressed simultaneously [26,29].

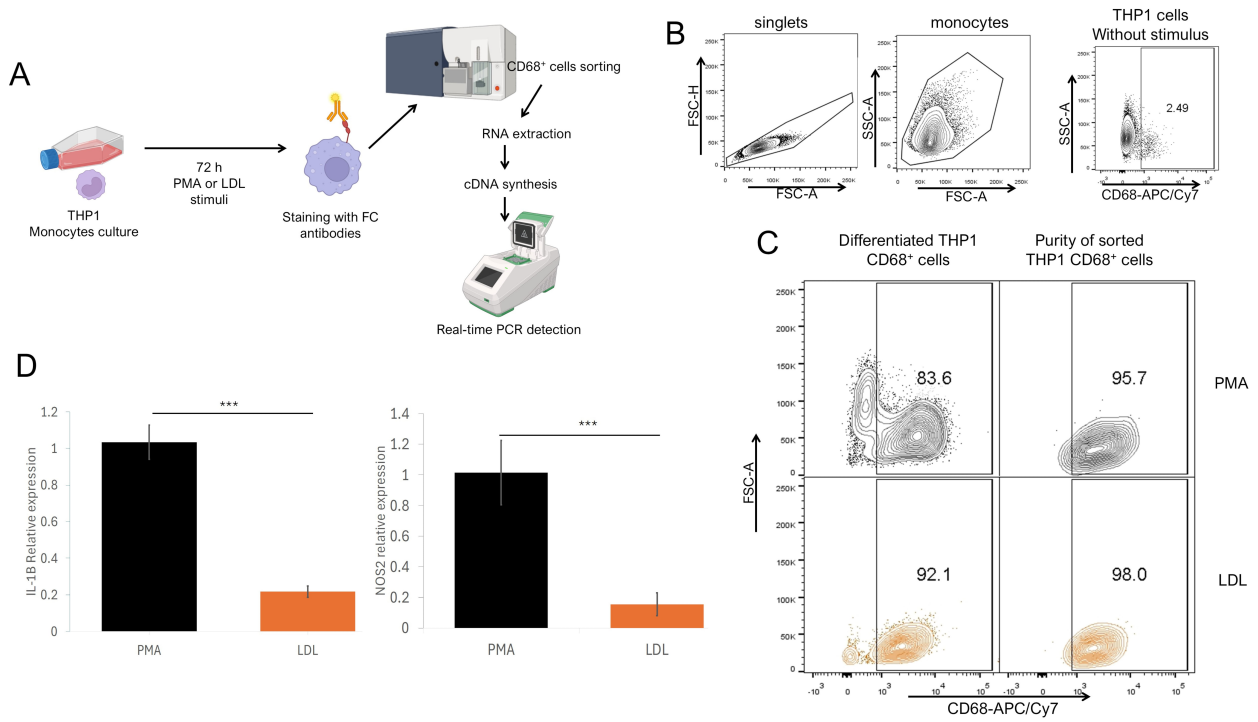

Fig. 4. M1 gene profile in THP1 CD68$^+$ macrophages stimulated with native LDL. (A) Experimental strategy. THP1 cells were cultured and stimulated for 72 h with PMA or LDL, and stained with anti-CD68 fluorochrome-coupled antibodies and sorted by flow cytometry. CD68$^+$ sorted cells were processed for RNA extraction and cDNA synthesis for qPCR detection. (B) Analysis strategy for sorted THP1, using unstimulated THP1 as a control where singlets were selected after stimulated monocytes were defined by their FSC-A SSC-A characteristics to sort CD68$^+$ cells vs. SSC-A characteristics. (C) Total differentiated cells percentage (left) and purity of sorted (right) cells stimulated with PMA (upper) or LDL (lower). (D) Relative *IL-1β* and *NOS2* expression in THP1 monocytes under LDL stimulus (orange). All relative expression was compared with the expression levels in the PMA condition. Data are presented as individual values with a median and range from two independent experiments performed in triplicate. ***$p = 0.00048$ for *IL-1β* relative expression and ***$p = 0.000976$ for *NOS2* expression. qPCR, quantitative PCR; NOS2, nitric oxide synthase 2.

Our results strongly showed that when THP1 cells were exposed for 72 h to LPS, PMA, or IFN-γ in a controlled *in vitro* context, these individual stimuli promoted the activation of CD14$^+$CD16$^+$ monocytes towards a pro-inflammatory profile. Meanwhile, PMA and IFN-γ allowed for the slight differentiation of monocytes into CD68$^+$ macrophages. Interestingly, individual stimulation for 72 h with either native LDL or ox-LDL enabled us to efficiently activate THP1 monocytes, expressing CD14 and CD16 molecules and adopting a pro-inflammatory profile characterized by TLR2 expression and reduced expression of the suppression-associated marker PD-L2. Also, native LDL promotes monocyte differentiation into CD68$^+$ macrophages even more efficiently than PMA under these individual conditions. When we analyzed *iNOS* and *IL-1β* gene expressions in sorted macrophages stimulated with LDL, we observed a slight increase in those genes, adding evidence to the M1 profile with native LDL stimulation, but also suggesting that other signaling processes are likely necessary to induce the total effector function of M1 macrophages producing iNOS and IL-1β. Some *in vitro* and *in vivo* models had been shown that both IL-1β and iNOS from macrophages have a role by directly promoting inflammation in an obese context [30,31]. However, a recent study suggests that IL-1β from tissue-resident macrophages has a pro-adipogenic but not pro-inflammatory role [32]. In the future, it will be interesting to evaluate whether CD68$^+$ macrophages stimulated with LDL in combination with some obesity-derived molecules have similar effector functions to those of pro-inflammatory macrophages, producing iNOS, inducing phagocytosis, and secreting pro-inflammatory cytokines, which can be evaluated in cultured supernatants.

On the other hand, when monocytes were pre-stimulated with PMA for 48 h and subsequently stimulated with obesity-associated biomolecules such as LDL, ox-LDL, and PGE2, we observed improved differentiation into CD68$^+$ macrophages under all evaluated conditions. These macrophages exhibited a dual activation phenotype. This finding, despite its *in vitro* approach, better represents the frequent conditions encountered by monocytes in an ordinary *in vivo* context, where a wide variety of stimuli collectively define the activation profile of immune cells.

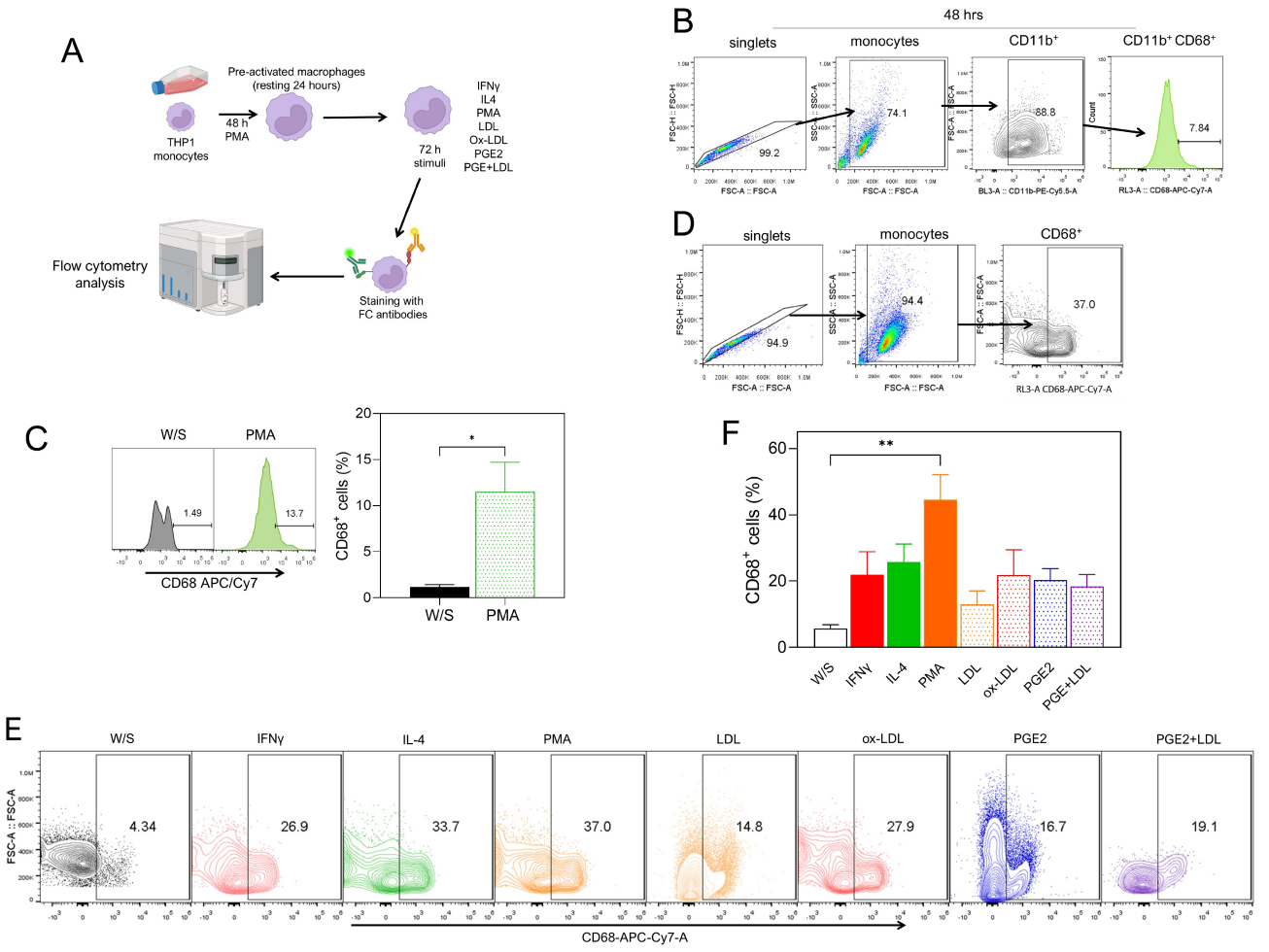

Fig. 5. Native LDL, ox-LDL, and PGE2 induced increased differentiation of PMA-preactivated THP1 monocytes to CD68$^+$ macrophages. (A) Experimental strategy. THP1 cells were cultured for 48 h with PMA, washed and rested for 24 h, and finally stimulated for 72 h with IFN-γ, IL-4, PMA, LDL, ox-LDL, or PGE2 for staining with fluorochrome-coupled antibodies and analysis by flow cytometry. (B) Analysis strategy for CD11b$^+$ and CD68$^+$ cells after 48 h of PMA stimulation, where singlets were selected after stimulated monocytes were defined by their FSC-A SSC-A characteristics to select CD11b$^+$ cells expressing CD68 marker. (C) Representative (left) and total (right) CD68 percentage in cells cultured for 48 h with PMA, *p = 0.0349. Three different experiments were performed in duplicate. (D) Analysis strategy for CD68$^+$ cells stimulated according to described in Fig. 5A. The percentage was obtained by flow cytometry, where singlets were selected, then monocytes were defined by their FSC-A and SSC-A characteristics. Finally, CD68$^+$ cells were selected. (E) Representative and (F) Total CD68$^+$ percentage in the groups with different stimuli by flow cytometry from four independent experiments performed in duplicate, **p = 0.0081. Statistical differences were assessed by one-way ANOVA followed by Dunnett's multiple comparison test with unstimulated cells serving as the control group.

A homogeneous or singular stimulation does not exist under ordinary stimulus conditions, such as inflammation. Instead, the immune response faces varied mixed stimuli, such as cytokines, pathogens, and damaged-associated molecular patterns, which are potent stimulants for differentiating innate immune cells [33]. Likewise, how innate immune cells are activated and differentiated directly affect the adaptive immune response that occurs, promoting exacerbated or chronic pro-inflammatory processes and even immunosuppression [34]. During chronic inflammatory processes such as low-grade inflammation induced by overweight and obesity, the native biomolecules LDL and PGE2, as well as biomolecules modified by inflammatory processes like ox-LDL, which are present in both the inflammatory microenvironment and the adipose tissue associated with inflammation, can favor the activation and differentiation of immune cells [35]. A spotlight has been placed on the capacity of the adipose microenvironment to influence the differentiation of adaptive immune cells [35]. Still, it is important to focus on the response capabilities of innate cells, such as monocytes and macrophages. Recently, it has been suggested that the exposure of macrophages to lipid products from obesity can affect their activation and differentiation and play a rele-

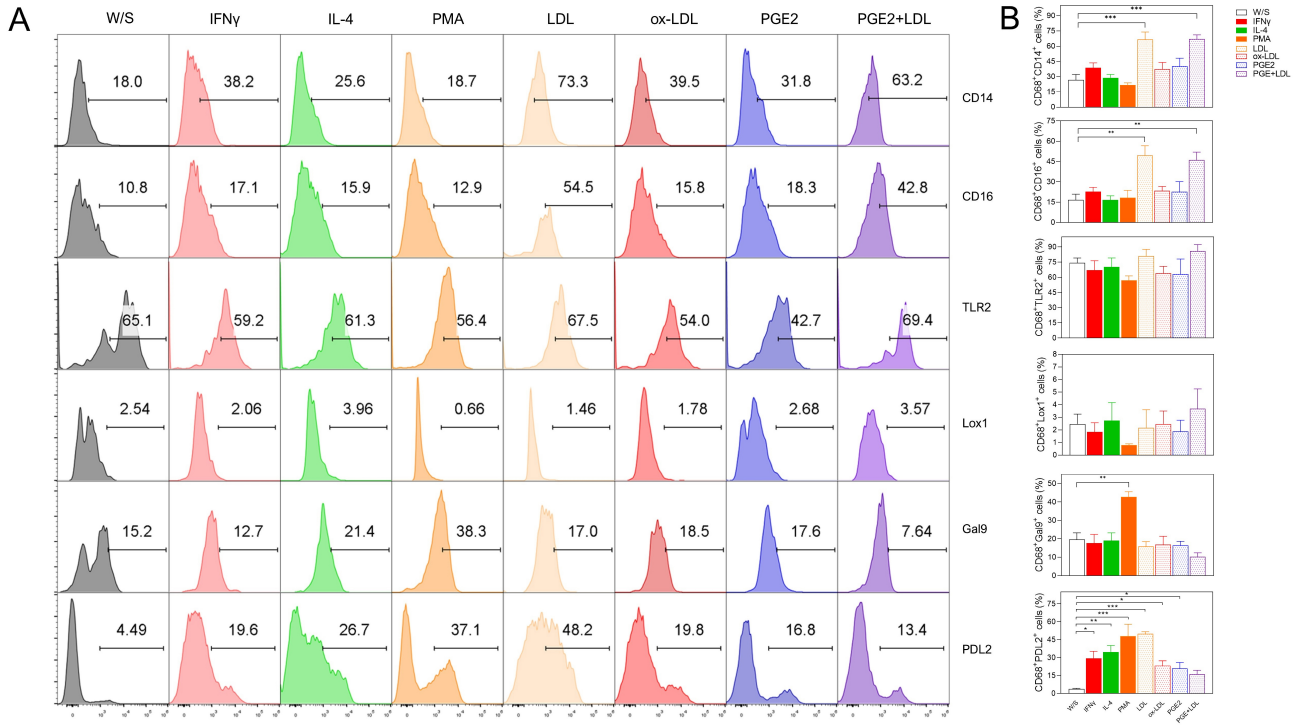

Fig. 6. Native LDL, ox-LDL, and PGE2 induced a dual activation profile in PMA-preactivated THP1 CD68$^+$ macrophages. THP1 cells were cultured for 48 h with PMA, washed and rested during 24 h, and finally stimulated for 72 h with IFN-γ, IL-4, PMA, LDL, ox-LDL, or PGE2. (A) Representative and (B) Total percentage of CD68$^+$ cells in the groups with different stimuli by flow cytometry from five independent experiments performed in duplicate. Statistical differences were assessed by one-way ANOVA followed by Dunnett's multiple comparison test with unstimulated cells serving as the control group. ***$p = 0.0002$ in CD68$^+$CD14$^+$ population for LDL and ***$p = 0.0002$ in CD68$^+$CD14$^+$ population for PGE2+LDL; **$p = 0.0010$ in CD68$^+$CD16$^+$ population for LDL and **$p = 0.0030$ in CD68$^+$CD16$^+$ population for PGE+LDL; **$p = 0.0015$ in CD16$^+$Gal9$^+$ population; in CD68$^+$PD-L2$^+$ population: *$p = 0.0303$ for IFN-γ, **$p = 0.0070$ for IL-4, ***$p = 0.0002$ for PMA, ***$p = 0.0003$ for LDL, *$p = 0.0130$ for ox-LDL and *$p = 0.0216$ for PGE2.

vant role in the homeostatic metabolism of lipids, which could trigger systemic inflammatory processes [36]. As previously mentioned, ox-LDL is involved in the monocyte to macrophage differentiation process during atherosclerosis [7]. However, even the images shown by the authors, data were obtained classifying macrophages with CD11b myeloid marker, which is expressed in a wide range of immune cells including both monocytes and macrophages [37]. It is necessary to comprehensively characterize samples from patients who developed diseases associated with obesity to have a better approach to immune cell profile activation. Our study was limited to controlled *in vitro* variables; however, it could be interesting to characterize the immune cells in obese human samples, probably by analyzing data from single-cell RNA sequencing with flow cytometry data validation. Recently it was demonstrated by single-cell RNA sequencing that triggering receptor expressed on myeloid cells 2-positive (TREM2$^+$) macrophages play a major role in inducing protection from kidney injury in obese patients [38]. On the other hand, obesity-derived biomolecules could have an infinity loop effect over immune cell differentiation, inducing pro-inflammatory phenotypes associated with the reactive oxygen species (ROS) overproduction, which in turn induces LDL oxidization [8]. Our results add evidence to the pro-inflammatory role of ox-LDL in THP1 monocytes. It has been shown with a different re-stimulation protocol using LPS over human monocytes that ox-LDL induces a pro-inflammatory profile with IL-6 and TNF-α production [39]. However, its role in macrophages is different, because we observed that CD68$^+$ macrophages have a dual phenotype with ox-LDL addition. It is likely that there is a different expression of pattern recognition receptors during the monocyte-macrophage activation and differentiation transition, explaining those differences.

The ability of obesity-associated molecules to induce monocyte activation or macrophage differentiation, as shown in this work, is because immune cells possess a broad range of pattern recognition receptors such as TLRs, NOD-like receptors, RIG-I-like receptors (RLRs), C-type lectin receptors, and scavenger receptors, among others. Once these receptors interact with their ligands, they trigger the activation and effector function of the immune cell [40]. PGE2 can be recognized by prostanoid receptors EP1

to EP4, which are constitutively expressed in mast cells and T lymphocytes. In fact, it is strongly suggested that PGE2, when bound to EP receptors expressed in T lymphocytes, can influence the adaptive response profile they present, whether it is T helper 1 (Th1) or Th17 cells [41]. Additionally, the prostanoid receptor EP4 is expressed in macrophage subpopulations in the intestine, promoting mucosal repair [42]. However, it remains unclear whether these prostanoid receptors are constitutively expressed in monocytes or macrophages. This might explain why we did not find a significant effect of PGE2 stimulation on THP1 monocyte cultures. On the other hand, the role of LDL in macrophages is to transform into foam cells associated with adipose tissue [43,44] and macrophages internalize LDL through pinocytosis or receptor-mediated phagocytosis via the LDL receptor [43], requiring pre-stimulation with PMA [44]. Although it is known that LDL can induce the differentiation of blood monocytes into macrophages [11], to the best of our understanding, this is the first report showing that LDL can differentiate monocytes into macrophages with an M1 activation phenotype without PMA pre-stimulation, as demonstrated by the higher percentage of CD68 and CD16 markers, and the expression of *IL-1*β and *iNOS* gene. Furthermore, ox-LDL is a molecule recognized by a wide range of innate immune response receptors, such as class A scavenger receptors like CD36 or class D scavenger receptors like CD68 and the LOX-1 receptor. LOX-1 expression is an indicator of endothelial cell dysfunction, and ox-LDL can promote the transformation of macrophages into foam cells through both the CD36 receptor [45] and LDL receptor in the murine model [43]. Additionally, during cancer, the LOX-1 receptor is overexpressed in peripheral blood neutrophils, inducing a suppression-associated phenotype known as myeloid-derived suppressor cells [46]. Recently, it was shown that $CD4^+$ T lymphocytes can recognize ox-LDL through the CD69 receptor due to its high homology with the LOX-1 receptor, which influences the differentiation processes toward a regulatory T cell profile inhibiting inflammatory profiles of Th17-associated response [47]. Together, these findings suggest that the obese microenvironment not only has an effect on immune cells but also contributes to the development of chronic diseases like cancer and atherosclerosis.

5. Conclusions

Our data suggest that native LDL induces the activation of THP1 monocytes and its differentiation into M1 macrophages even more efficiently than classic PMA stimulation, and ox-LDL favors the monocyte activation profile. On the other hand, ox-LDL and PGE2 induce the expression of activation markers similarly to IFN-γ or IL-4 in macrophages pre-activated with PMA. Also, our work suggests for the first time that both stimuli, PMA and obesity-associated molecules, induce expression of the LOX-1 receptor in monocytes and macrophages. Our future perspectives include evaluating the effector capacity of macrophages differentiated with obesity-associated biomolecules to show their functional ability in either pro-inflammatory- or suppressive-associated profiles. If those macrophages have an M1-related profile, we will show their capacity to induce phagocytosis, ROS production, and inflammatory cytokines as effector function; however, if the function is M2-related, we will evaluate their immunosuppressive capacity over the proliferation of T cells. Also, we are interested in performing single-cell RNA sequencing in myeloid cells from patients diagnosed with obesity. Finally, the common and constant stimuli (in this case, associated with obesity) faced by cells of both the innate and adaptive immune response promote both their differentiation and the type of activation profile displayed, which may be crucial in shaping the immunity that will protect the body from chronic degenerative diseases. We strongly suggest that the conditions allowing immune cell differentiation in a chronic inflammatory context, such as obesity-associated biomolecules, are crucial to enabling deficient immunosurveillance and promoting immunosuppression, which are observed in some pathologies like colorectal cancer. There is still a long way to go before tangible evidence is obtained regarding the role that obesity-derived biomolecules play in chronic-degenerative diseases; however, the findings from this study are the first steps suggesting that LDL, ox-LDL and PGE2 play an important role in defining the profile of innate immune cells.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conception and experimental design: JEO, LIT, VGA and VGG. Experimental performance: JEO, VGA, VGG, VHG, MGM and NSJ. Data analysis: VGA, JEO, LIT, MGM and VGG. Interpretation of the results: JEO, VGA, VGG, MGM, and NSJ. Paper writing: JEO and LIT. Funding resources: JEO and MGM. All authors contributed to editorial changes in the manuscript. All the authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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