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Secreted phospholipase A_2 type IIA as a mediator connecting innate and adaptive immunity: new role in atherosclerosis

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Aims Human atherosclerotic plaques express markers of macrophage/dendritic cells as well as high levels of inflammatory proteins such as secreted phospholipase A_2 type IIA ($sPLA_2$-IIA). To understand the cellular changes associated with the progress of atherosclerosis, we evaluated the role of $sPLA_2$-IIA in mediating monocyte recruitment and differentiation into antigen-presenting cells.
Methods and results The effect of $sPLA_2$-IIA on monocyte differentiation was evaluated in human THP-1 cells, a cellular line widely used as a model for monocyte–macrophage differentiation. Changes in functional processes, morphology and expression of antigens, characteristic of differentiated cells, were monitored over a 1–3 day period. $sPLA_2$-IIA inhibited CD14 expression in a time- and concentration-dependent manner and upregulated dendritic cell-specific ICAM-3 grabbing non-integrin levels at the cell surface, findings that were the same for human monocytes. In addition, $sPLA_2$-IIA-differentiated cells showed a dendritic cell phenotype characterized by the generation of fine dendritic protrusions and an increase in surface markers such as CD40, CD83, CD54, CD61, and CD62L. Furthermore, cell adhesion, migration, endocytic activity, and allogeneic T cell proliferation capacity were markedly increased after $sPLA_2$-IIA treatment.
Conclusion $sPLA_2$-IIA induces the differentiation of mononuclear cells and increases their adhesive and migratory capabilities, which suggests a novel function for $sPLA_2$-IIA as a mediator connecting innate and adaptive immunity. These findings may provide insight into the immuno-inflammatory processes occurring in atherosclerosis, helping us to understand the cellular changes associated with the development of atherosclerosis.

1. Introduction

Atherosclerosis is a complex process characterized by lipid accumulation in the arterial wall, and it is accompanied by a local inflammatory response in which innate and adaptive immune mechanisms play key roles in plaque formation.[1–3] The inflammatory response is mediated by functional changes in endothelial cells, T lymphocytes, monocytes/macrophages, and smooth muscle cells (SMC).[4] Recently, dendritic cells (DCs), derived from monocyte precursors, have also been reported to participate in the atherosclerotic process by activating T cells.[5,6] Their occurrence in atherosclerotic lesions, as well as in the healthy intima, predisposes them to the development of atherosclerosis, as it has been demonstrated in both human and animal models.[7–9] In keeping with this, the expression of DC markers, including CD80 or DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin), has been shown in human atherosclerotic plaques.[10] And, since these markers mediate adhesion and endocytosis, a fundamental aspect of DC function, it has been suggested that their expression may potentially have critical functional implications.

In their immature form, DCs are equipped with receptors to become activated when exposed to pathogen-associated molecular patterns. After antigen uptake, DCs migrate and differentiate to phenotypically and functionally mature DCs. These DCs, depending on the maturation state and the local microenvironment, may act to induce immunological tolerance and/or regulation of Tcell-mediated immunity.[11–13]

A multitude of signals, including bacterial components such as LPS, or pro-inflammatory factors such as TNFα or IL-1β, can promote the maturation of DCs inducing a switch from an immature stage to a stage of strong T cell-stimulatory capacity.[14] Current studies with enzymes from bee venom, such as secreted phospholipase A_2 type III ($sPLA_2$-III), have indicated that $sPLA_2$-III can also act on DC

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membranes mobilizing lipid mediators and inducing their maturation.[15]

Mammalian-secreted PLA$_2$s present many catalytic and structural similarities with venom phospholipases, they are extracellular low-molecular-mass (13–18 kDa) enzymes that have 6–8 disulphide bridges, and require millimolar concentrations of Ca^{2+} for enzymatic activity.[16] The plasma of patients with various inflammatory diseases, particularly atherosclerosis, contains high concentrations of the isoform sPLA$_2$-IIA, and clinical studies reveal a correlation between circulating levels of the phospholipase and vascular complications.[17]

sPLA$_2$-IIA, which can be upregulated by inflammatory cytokines, such as IL-1β, IL-6, and TNFα, acts as an acute phase reactant in chronic inflammatory diseases and it is clearly linked to the development of atherosclerotic lesions.[18,19] It has been detected in atherosclerotic arteries and may trigger its effects on all the atheroma-associated cells. sPLA$_2$-IIA is present in VSMC from both media and intima layers and in macrophage-rich regions of atherosclerotic plaques, and it may induce foam cell formation and low-density lipoprotein modification.[20,21] To date, details of sPLA$_2$-IIA actions and its precise physiological and/or pathophysiological role have not yet been defined. Although this enzyme is involved in the specific synthesis of lysophosphatidic acid, unesterified fatty acids, and prostaglandins, and most of its biological activities have been attributed to its capacity to generate biologically active lipids, which may sustain inflammation, several lines of evidence suggest a mechanism of action involving the human M-type receptor, or a related structure, as the mediator of its effects.[22–24]

In this work, we examined the role of sPLA$_2$-IIA in monocyte biology and we found that sPLA$_2$-IIA can act on THP-1 cells to induce a monocyte-derived dendritic cell (MDDC) phenotype, emphasizing the role of this secreted enzyme in the regulation of immune responses and its implication in atherosclerotic processes.

2. Methods

2.1 Materials

A C127 mouse fibroblast cell line stably transfected with the coding sequence of sPLA$_2$-IIA from human placenta was used as a source of human recombinant enzyme. sPLA$_2$-IIA was purified as described previously.[23] FITC-phalloidin, FITC-dextran, PMA, and other chemicals were from Sigma Chemical Co. (St Louis, MO, USA). Recombinant TNFα and IL-4 were from Calbiochem. Monoclonal anti-human DC-SIGN was a generous gift from Dr A.L. Corbí (Centro de Investigaciones Biologicas, CSIC, Madrid, Spain).

2.2 Cell culture

THP-1 cells were cultured at 37°C under 5% CO_2 atmosphere in RPMI 1640 (GIBCO) with 100 U/mL penicillin/streptomycin, 2 mM glutamine, and 5% FCS and maintained around 5×10^5 cells/mL.

2.3 Cell isolation from peripheral blood and monocyte-derived dendritic cell generation

Human monocytes were purified and differentiated according to standard procedures.[25] Briefly, peripheral blood mononuclear cells were isolated from fresh buffy coats (provided by the Center for Hemotherapy of Castilla y León) by lymphoprep density-gradient centrifugation. Then, cells were washed and centrifuged at 1500 r.p.m. to avoid platelets. After 1 h of plastic adherence at 37°C in RPMI 1640 with 10% FCS, non-adherent cells were removed by various vigorous washes with PBS and used as T cells in the mixed-leucocyte reaction (MLR) assay. The remaining adherent cells were subsequently differentiated with 1 μg/mL sPLA$_2$-IIA or 0.2 μM of PMA + 1000 U/mL of IL4 for the indicated days.

2.4 FITC-dextran uptake by mononuclear cells

Human THP-1 cells or human monocytes were incubated with vehicle, 1 μg/mL sPLA$_2$-IIA, 0.2 μM of PMA, or 0.2 μM of PMA + 1000 U/mL of IL4. After 72 h, cells were suspended in culture medium with 1 mg/mL FITC-dextran (MW 40 000) and incubated at 4°C (for background control) or at 37°C for 30 min. Then, cells were washed with cold PBS containing 1% FCS, and the FITC-dextran incorporated was analysed by flow cytometry in an EPICS® XL-MCL cytofluorometer (Coulter). The endocytic activity is expressed as the mean fluorescence intensity (MFI) index. MFI index is calculated as fluorescence of FITC-positives cells at 37°C minus fluorescence of FITC-positive cells at 4°C and normalized at 100 for the control.

2.5 Flow cytometry

THP-1 cells were analysed for CD 11b, CD11a, CD11c, CD1a, CD83, CD40, CD14, CD61, CD62L, DC-SIGN, or ICAM expression (Pharmingen, San Diego, CA, USA). Control and treated cells were collected for immunolabelling with 10 μg/mL of each indicated antibody for 1 h at 4°C, followed by PE- or FITC-conjugated secondary antibody (Sigma). Subsequently, cells were washed, resuspended in 500 μL of PBS and analysed by flow cytometry. Positive cells were estimated using P3-X63 myeloma supernatant as a negative control or an isotype-matched control-negative antibody at an equivalent concentration. Data analysis was performed using WinMDI 2.7 software.

2.6 THP-1 cell morphology

THP-1 cells were seeded on glass coverslips (1.2×10^4 cells/slip). Then, cells were stimulated for 72 h. After that, cells were washed with PBS, fixed for 10 min with 3.7% formaldehyde/PBS, and permeabilized for 5 min with 0.3% Triton X-100/PBS. Actin morphology was assessed by staining with FITC-phalloidin and was visualized using a Bio-Rad laser scanning Radiance 2100 system coupled to a Nikon Eclipse TE-2000U confocal microscope with a $\times$60 oil objective.

2.7 Assessment of morphological changes and adhesion assay

Under normal culture conditions, THP-1 cells remain in suspension; however, after treatment with sPLA$_2$-IIA, a subpopulation of cells become adherent. Morphological changes of the adherent cells were assessed by phase-contrast microscopy. In addition, adherent cells were quantified by using the [methyl-^{3}H]-thymidine-labelled adhesion assay. Briefly, THP-1 cells (5×10^6) were incubated in RPMI/5% FBS in the presence of [methyl-^{3}H]-thymidine (Amersham, UK) at a final concentration of 0.5 μCi/mL at 37°C for 18 h. After that, cells were washed with PBS and resuspended in RPMI and then used in the adhesion assay.

The labelled cells were stimulated with 1 μg/mL of sPLA$_2$-IIA, 0.2 μM of PMA, or 0.2 μM of PMA + 1000 U/mL IL-4 at 37°C for the indicated times. Then, the plates were rinsed four times with PBS, adherent cells were scraped, and the amount of radioactivity present was determined by liquid scintillation counting.

2.8 Chemotaxis assay

Chemotaxis assay was performed in a six-well plate using tissue culture polycarbonate filter inserts (3 or 8 μm pore, Corning, Costar, Cambridge, MA, USA) blocked with BSA. THP-1 cells (5×10^6) were resuspended in 1 mL of serum-free medium, loaded into the upper chamber of an insert, and incubated at 37°C. The lower chamber contained conditioned medium from cells incubated with either vehicle, 100 U/mL of TNF-α, or 1 μg/mL of sPLA$_2$ for 18 h.

After 45 min, the membrane was removed, and the cells in the lower chamber were recovered and counted in a cytofluorometer. The results are expressed as the fold increase over the control.

2.9 Allogeneic mixed-leucocyte reaction: T cell proliferation assay

The immunostimulatory capacities of the $sPLA_2$-IIA-treated THP-1 cells to activate T cells was measured in an MLR and quantified by the MTS assay (Cell Tilter 96TM Non-Radioactive Cell Proliferation Assay, Promega, Inc.) method, according to the manufacturer's recommendations.

THP-1 cells were differentiated for 5 days with 1 μg/mL $sPLA_2$-IIA or 0.2 μM of PMA + 1000 U/mL of IL4 (effector cells) and treated with 50 μg/mL mitomycin C (Sigma) for 20 min at 37°C to inhibit cell proliferation. After extensive washes, various numbers (10^3, 5×10^3, 10^4) of these differentiated THP-1 cells (THP-1-DC) were added to freshly isolated 10^4 lymphocytes (responder cells). Triplicates of the co-culture were incubated for 3 days in 96-well round-bottom microtitre plates, and the proliferative T cell response was quantified by the addition of MTS solution (20 μL/well). After 1 h incubation, the conversion of MTS to formazan was measured at 490 nm in a microplate reader (VERSAmax, Molecular Devices, CA, USA). As control, lymphocytes were cultured in the absence of THP-1-DC. For data analysis, the actual absorbance values from the co-culture were adjusted for the background absorbance of THP-1-DC. Proliferation index was calculated as the ratio of the corrected absorbance for a specific experimental condition to the absorbance of control culture (lymphocytes alone).

2.10 Data presentation

Numerical data are expressed as means ± SD and analysed with the GraphPad Prism Version 4 software (San Diego, CA, USA) for statistical significance using the one-way ANOVA test, followed by Dunnett's multiple comparison test to compare differentiated cells to untreated control cells. $P < 0.05$ was considered significant.

3. Results

3.1 Secreted phospholipase A_2 type IIA promotes THP-1 monocyte differentiation

We studied the capacity of $sPLA_2$-IIA to modulate monocyte differentiation into either macrophages or DCs. In order to

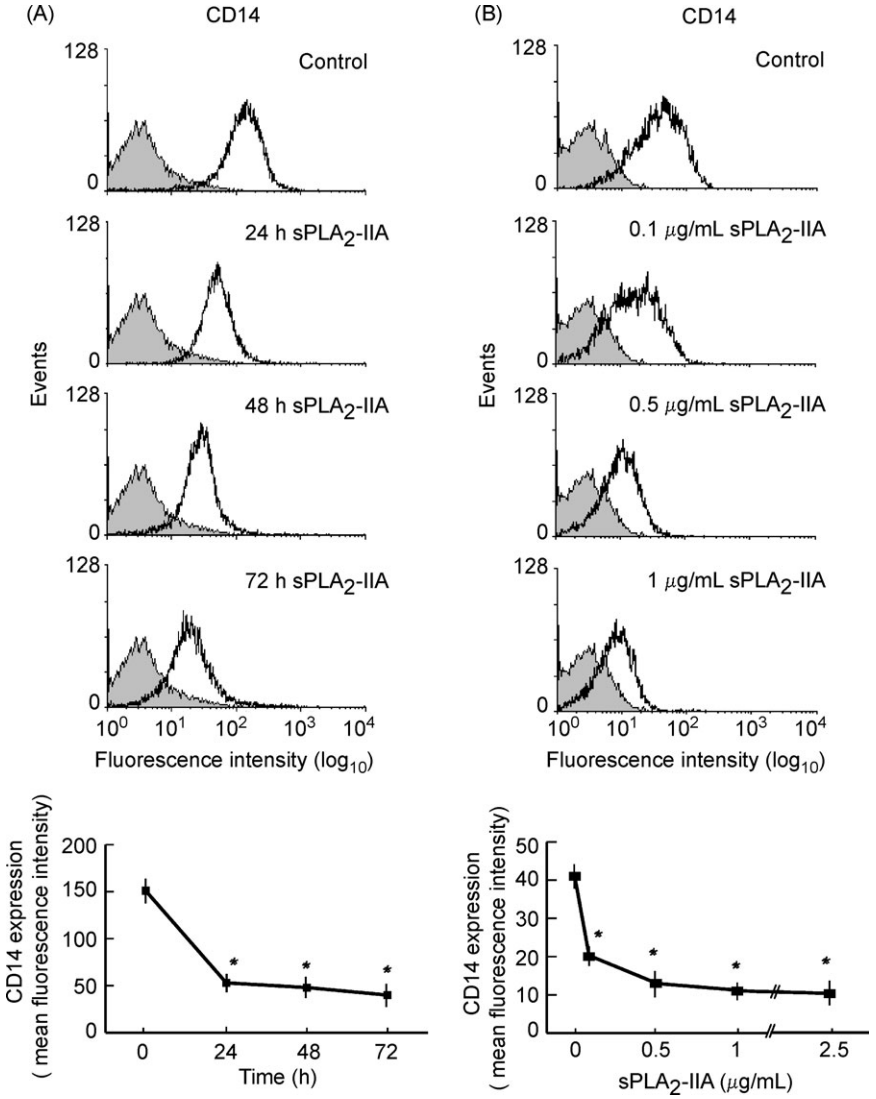

Figure 1 Secreted phospholipase A_2 type IIA ($sPLA_2$-IIA) reduces the expression of CD14 on THP-1 cells. Cells were treated with 1 μg/mL of $sPLA_2$-IIA for various time periods at 37°C (*A*) or for 48 h with different doses of $sPLA_2$-IIA (*B*), and CD14 expression was analysed by flow cytometry (empty black curves) and compared with isotype controls (solid grey curves). Histograms represent one experiment out of three. The binding of anti-CD14 is expressed as mean fluorescence intensity. Graphics, mean of three independent experiments ± SD; $n = 3$. *$P < 0.01$ vs. control untreated cells.

characterize the phenotypic modifications elicited by sPLA$_2$-IIA, we used a well-characterized human promonocytic cell line, THP-1, since these cells have been extensively considered a valuable model system for monocyte–macrophage differentiation and for studying intracellular sPLA$_2$-IIA-signalling pathways. Thus, THP-1 cells were stimulated with the phospholipase, and the expression of CD14 was analysed in the culture after 24, 48, and 72 h by flow cytometry. As shown in *Figure 1A*, the treatment with 1 μg/mL sPLA$_2$-IIA triggered a time-dependent downregulatory effect on CD14 surface expression. To define the dose-dependency of the action of the sPLA$_2$-IIA, cells were incubated for 24 h with different concentrations of sPLA$_2$-IIA. *Figure 1B* shows the effects of the different concentrations of sPLA$_2$-IIA on CD14 downregulation. The optimal effect took place at a concentration of 1 μg/mL sPLA$_2$-IIA, which is the concentration used in experiments to follow.

Apart from CD14 downregulation, also morphological changes in THP1 can be observed after incubation with the phospholipase. *Figure 2A* illustrates cellular morphology studies under phase-contrast microscopy, where it is shown that, in the presence of the phospholipase, cells became adherent, formed homotypic aggregates, and displayed cytoplasmic protrusions, characteristic morphology of DCs. Cell clustering began 24 h following sPLA$_2$-IIA stimulation and was further enhanced after 48–72 h. We also incubated THP-1 cells with PMA + IL-4, known to induce phenotypic and functional MDDCs. As shown in *Figure 2A*, this treatment yielded similar results to those found with the phospholipase. Moreover, cells were stained for F-actin and observed under confocal (*Figure 2B*, upper panels) or conventional (*Figure 2B*, lower panels) fluorescence microscope; the studies revealed that sPLA$_2$-IIA-treated THP-1 cells also developed morphological changes suggestive of MDDC, since they exhibited numerous projections and a laterally positioned nucleus, similar to what it is induced after PMA + IL-4 treatment. In addition, the modulation of CD14 was also evaluated in fluorescence immunostaining experiments. As shown in *Figure 2C*, the signal corresponding to CD14, constitutively expressed in control cells, was barely detectable after differentiation.

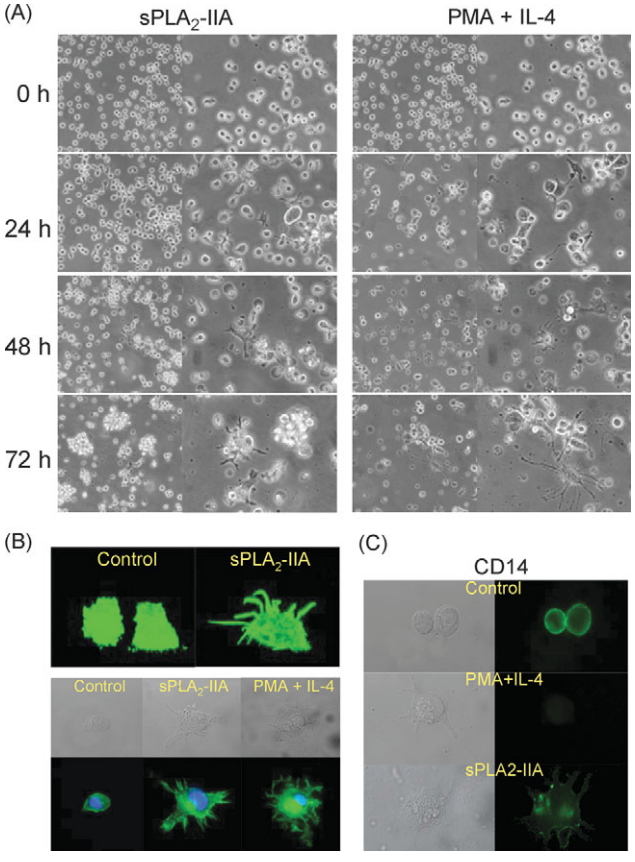

Figure 2 Secreted phospholipase A$_2$ type IIA (sPLA$_2$-IIA) affects the morphological appearance of THP-1 cells. Cells were plated onto poly-L-lysine-coated coverslips. After stimulation with 1 μg/mL secreted phospholipase A$_2$ type IIA or 0.2 μM PMA + 1000 U/mL IL4 for the indicated times, cells were fixed, and visualized under a phase contrast microscope (Nikon Eclipse TS100, ×20, ×40) (*A*), or labelled with FITC-phalloidin (*B*) or anti-CD14 antibody (*C*) and analysed with a confocal (*B*, upper panels) or a conventional (*B*, lower panels, and *C*) fluorescence microscope. Images are representative fields of cells stimulated for 72 h, the cellular morphology observed using Nomarski optics.

The effect found on CD14 surface expression after sPLA$_2$-IIA incubation (79% positive cells and 60 MFI) was similar to that obtained when the cells were incubated in the presence of the differentiation-inducing agents PMA and IL-4 (65% positive cells and 50 MFI), compared with untreated cells (98% positive cells and 175 MFI) (*Figure 3A*). Furthermore, DC-SIGN, the C-type lectin marker of DCs and MDDCs, barely detectable in resting THP-1 cells (15% positive cells and 20 MFI), was strongly expressed within 72 h of treatment with sPLA$_2$-IIA (82% positive cells and 52 MFI) in a manner comparable with what is observed after PMA and IL-4 (70% positive cells and 85 MFI).[25] Next, sPLA$_2$-IIA was added simultaneously to PMA and IL-4, and flow cytometry analysis revealed minor effects on both CD14 (62% positive cells and 47 MFI) and DC-SIGN (86% positive and 60 MFI) expressions, compared with the induction found after either the combination PMA+IL-4 or the phospholipase alone (*Figure 3A*). Interestingly, the same effect was also observed in human monocytes (*Figure 3C*). The modulation of DC-SIGN was also evaluated by western blot and by fluorescence immunostaining experiments. As shown in *Figure 3B*, the signal corresponding to DC-SIGN greatly increased in cells treated with either sPLA$_2$-IIA or PMA and IL-4 compared with untreated cells.

To go deeper into the phenotype of sPLA$_2$-IIA-treated THP-1 monocytes, flow-cytometrical analysis of monocyte/dendritic surface antigens was performed (*Figure 4*). The histograms demonstrate that after sPLA$_2$-IIA stimulation, cells share immunophenotypic features with PMA + IL-4-differentiated THP-1: CD14 was clearly downregulated and cells increased the level of the expression of the surface markers CD54 (ICAM-1), CD62L, and CD61, as well as the costimulatory molecules CD40 and CD83. However, although no significant differences were found in the presence of CD1a, CD18, CD11a, CD11b, or CD11c at any time point in sPLA2-IIA-treated cells, the presence of PMA + IL-4 led to an upregulation of CD11a, CD11b, CD1a, and CD18. In addition, neither the membrane mannose receptor (CD206) nor CD36, both markers of macrophages, were upregulated by the presence of sPLA$_2$-IIA (data not shown).

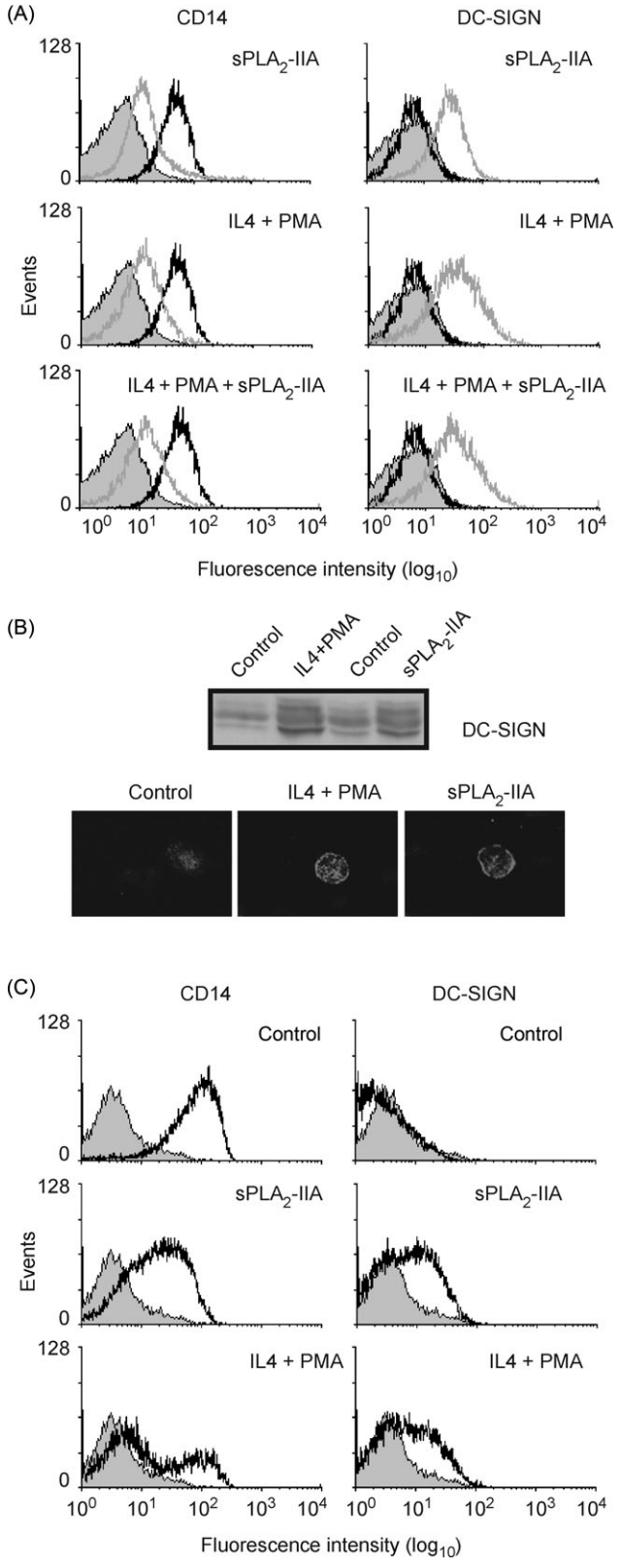

Figure 3 Secreted phospholipase A_2 type IIA ($sPLA_2$-IIA) upregulates dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) in human monocytes and in THP-1 cells. Cells were treated with 1 μg/mL secreted phospholipase A_2 type IIA, 0.2 μM PMA + 1000 U/mL IL4, or combination of both conditions for 72 h at 37°C. (*A*) Dendritic cell-specific ICAM-3 grabbing non-integrin and CD14 expressions in THP-1 cell were measured by flow cytometry. Solid grey curves represent unspecific binding; empty black curves, cells cultured in the absence of treatment (control); empty grey curves, agonist-treated cells. (*B*) THP-1 cells treated as above were either stained with antibodies against dendritic cell-specific ICAM-3 grabbing non-integrin and visualized under a fluorescent microscope (×60) or lysed and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot with an antibody against dendritic cell-specific ICAM-3 grabbing non-integrin. (*C*) Representative histograms of human monocytes exposed to 1 μg/mL secreted phospholipase A_2 type IIA or 0.2 μM PMA + 1000 U/mL IL4 for 72 h at 37°C. Treated cells (empty black curves) are compared with isotype controls (solid grey curves).

3.2 Effects of secreted phospholipase A_2 type IIA on functional cellular processes

3.2.1 Regulation of endocytosis by secreted phospholipase A_2 type IIA

Antigen uptake in immature DCs occurs primarily through endocytosis, and this ability is critical for the immunological function of these cells. However, this skill of efficient internalization of antigens is progressively lost during the maturational process, as mature DCs become focused on presenting antigens.[26] To determine the endocytic capacity of both $sPLA_2$-IIA-treated THP-1 cells (*Figure 5A* and *B*) and monocytes (*Figure 5C* and *D*), untreated and treated cells were incubated with the probe FITC-dextran, since it allows quantitation by flow cytometry analysis. *Figure 5A* and *C* shows FITC-dextran beads accumulation at 37°C in cells differentiated with the indicated agonist. Approximately 80% of the THP-1 cells became FITC-dextran-positive after 1 h of incubation with $sPLA_2$-IIA, and ~95% after PMA or PMA + IL-4 treatment. The narrow distribution of the fluorescence profile in the histograms indicates that all the cells of a particular condition take up comparable amounts of the probe; however, diverse MFI are observed between treatments owing to different efficiency in antigen capture. Similar behaviour was observed in monocytes, although in these cells the maximal response was found after PMA + IL-4 exposure. For statistical analysis, MFI values were normalized to 100 of control non-treated cells (*Figure 5B* and *D*).

3.2.2 Regulation of T cell stimulatory capacity: mixed lymphocyte reaction

Next, to evaluate the antigen-presenting function of $sPLA_2$-IIA-treated THP-1 cells, their ability to stimulate the proliferation of allogeneic lymphocytes was assessed by the MLR. As shown in *Figure 6A*, we found that $sPLA_2$-IIA-treated THP-1 cells were able to strongly stimulate the proliferation of lymphocytes, although their antigen-presenting ability was weaker than that of PMA + IL4-treated cells. This T cell stimulatory capacity of the differentiated THP-1 cells was in line with the phenotypic changes shown previously. The proliferative response rate correlated with the THP-1-DC/lymphocytes ratio. When untreated THP-1 cells were co-cultured with 10^4 lymphocytes at a ratio of 1:1, the proliferation index was 1.7 ± 0.3, meaning a 40 and 30% of the T cell proliferation induced by $sPLA_2$-IIA- or PMA + IL4-stimulated THP-1 cells, respectively, at the same ratio. Therefore, $sPLA_2$-IIA generates cells that possess the capacity to trigger functionally relevant interactions with T lymphocytes.

3.2.3 Regulation of chemotaxis by secreted phospholipase A_2 type IIA

The recruitment of DCs from the site of residence to the T cell areas is another essential event in the induction of

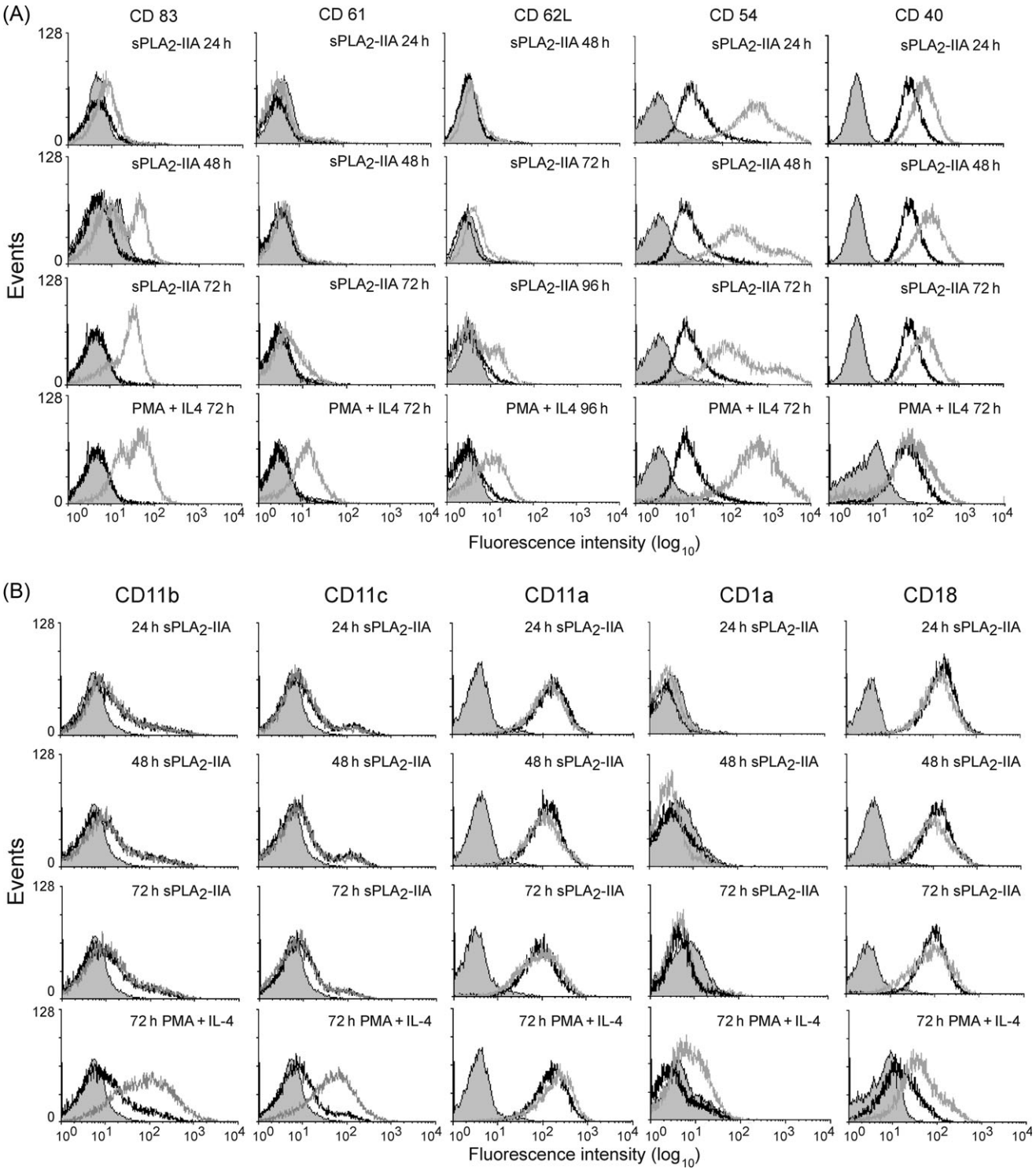

Figure 4 Phenotype of secreted phospholipase A_2 type IIA (sPLA$_2$-IIA)-treated cells. THP-1 cells were cultured in the presence of 1 μg/mL secreted phospholipase A_2 type IIA or 0.2 μM PMA + 1000 U/mL IL4 for different times. Histograms represent one experiment out of three and show the expression of the indicated surface markers analysed by flow cytometry. Solid grey curves represent unspecific binding; empty black curves, cells cultured in the absence of treatment (control); empty grey curves, agonist-treated cells.

primary immune responses, and several lines of evidence indicate that their migration is promoted by locally produced inflammatory cytokines such as TNFα.[27] To determine the migratory capacity of the cells in response to sPLA$_2$-IIA or to sPLA$_2$-IIA-produced factors, THP-1 cells were placed in the upper chambers of Transwell inserts. The lower chambers contained supernatants from non-stimulated cells, 24 h sPLA$_2$-IIA-stimulated cells, or 24 h TNFα-stimulated cells. With the 8 μm pore inserts, THP-1 cells showed a slight preferential migration in response to supernatants from sPLA$_2$-IIA-stimulated cells, over TNFα-supernatants, and migrated three-fold above the media

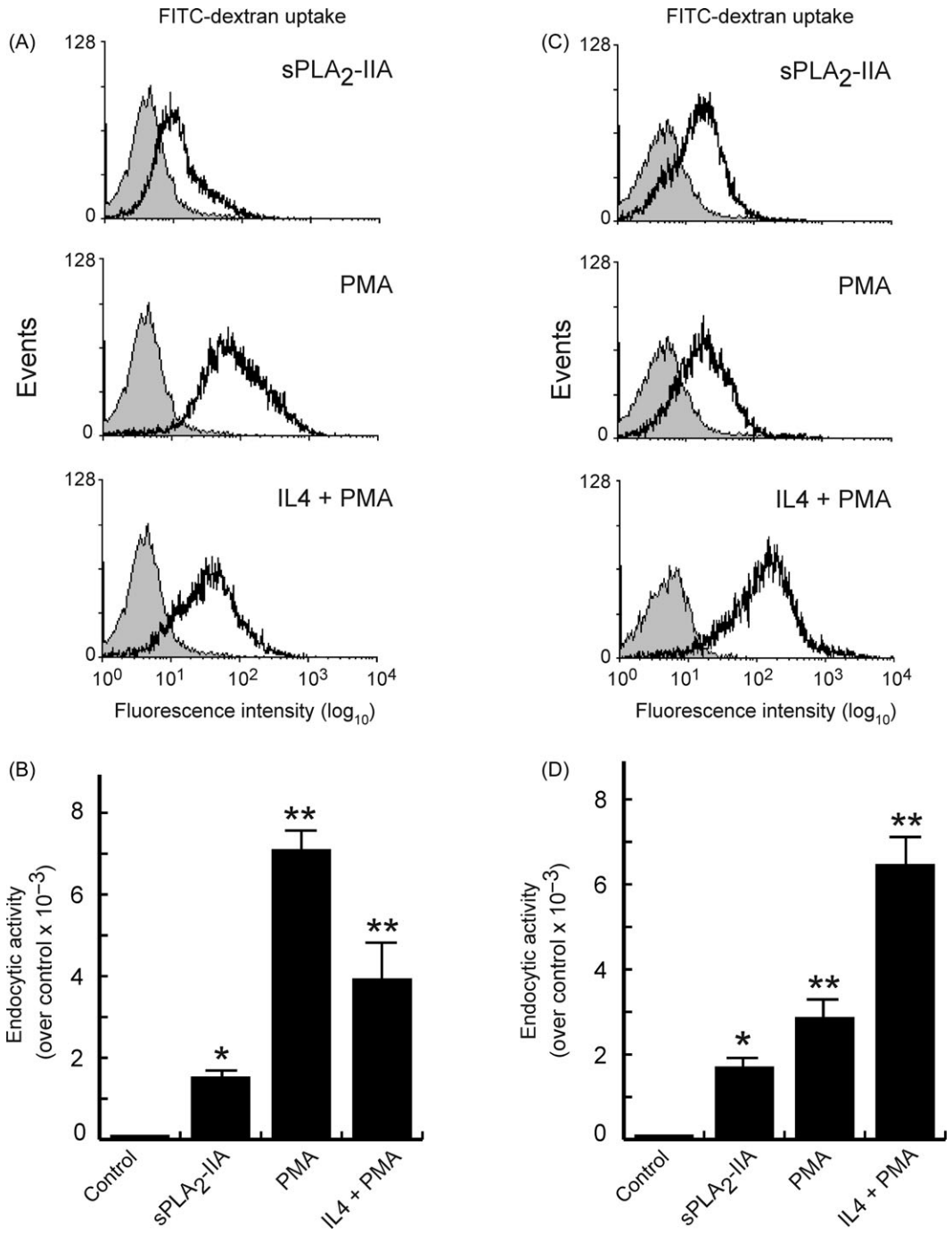

Figure 5 Endocytic capacity of secreted phospholipase A_2 type IIA ($sPLA_2$-IIA)-treated cells. THP-1 cells (*A* and *B*) and human monocytes (*C* and *D*) were cultured in the absence or presence of 1 μg/mL secreted phospholipase A_2 type IIA, 0.2 μM PMA, or 0.2 μM PMA + 1000 U/mL IL4 for 72 h. Then, they were incubated with FITC-dextran for 1 h and analysed by flow cytometry. (*A* and *C*) Histograms represent one experiment out of three. Solid grey curves represent unspecific endocytosis and empty black curves endocytosis of cells cultured as indicated. (*B* and *D*) Endocytic activity, as indicated in Methods, is expressed as mean fluorescence intensity index ± SD normalized by setting controls to 100%. $^*P < 0.05$, $^{**}P < 0.01$ vs. control untreated cells.

control (*Figure 6B*). With the 3 μm pore inserts, the migration of THP-1 cells exposed to $sPLA_2$-IIA supernatant was sensibly diminished, whereas those exposed to TNFα supernatants showed similar levels of chemotaxis to the 8 μm pore inserts. When the lower chambers contained media plus $sPLA_2$-IIA, no chemotactic effect was observed. In addition, we examined the migration of $sPLA_2$-IIA-treated cells exposed to $sPLA_2$-IIA supernatant, and we observed that there was no significant difference, compared with cells exposed to control media (data not shown).

3.2.4 Regulation of adherence by secreted phospholipase A_2 type IIA

The differentiation of monocytes to either macrophages or DC is accompanied by changes in their ability of adhesion to extracellular matrices. To test the ability of $sPLA_2$-IIA to affect cell adherence, [^{3}H]-thymidine-labelled THP-1 monocytes were aliquoted into six-well plastic plates and incubated for 12 or 24 h with the enzyme, PMA + IL-4, or PMA alone (as a reference agonist). As shown in *Figure 6C*, $sPLA_2$-IIA, as well as PMA or PMA + IL-4, was found to

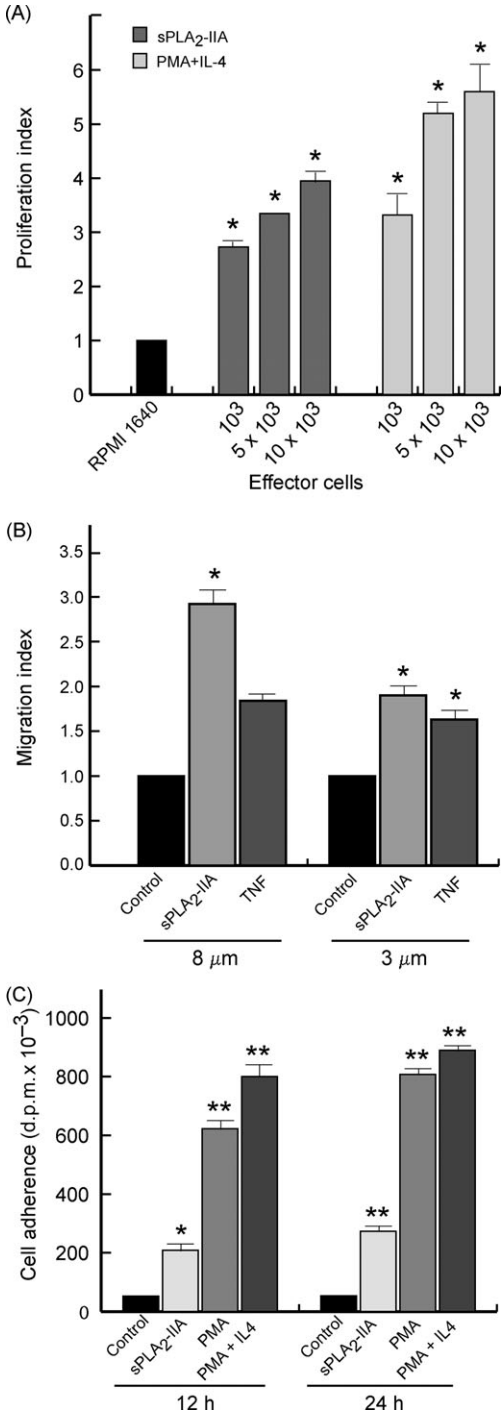

Figure 6 Effect of secreted phospholipase A_2 type IIA on functional capabilities of THP-1 cells. (*A*) T cell stimulatory activity. THP-1 cells were differentiated with 1 μg/mL secreted phospholipase A_2 type IIA or 0.2 μM PMA + 1000 U/mL IL4 for 5 days and treated with mitomycin C. Then, 10^4 peripheral blood T lymphocytes were cultured with RPMI alone or with various numbers of these mitomycin C-treated THP-1 dendritic cells. After a 3 day co-culture, proliferation was determined using the MTS assay, the proliferation index calculated as described in Methods. Optical density at 490 nm in the culture containing lymphocytes with RPMI alone (control) was 0.175 ± 0.032; $n = 3$. *$P <$ 0.001 vs. RPMI alone. (*B*) The migratory capacity was determined using 3 and 8 μm pore-size Transwell plaques, as indicated in Methods. The results are presented as a migration index denoting the fold increase of cell migration over control. *$P < 0.001$ vs. control. (*C*) THP-1 adherence was determined after 12 or 24 h stimulation with solvents (control), 1 μg/mL secreted phospholipase A_2 type IIA, 0.2 μM PMA, or 0.2 μM PMA + 1000 U/mL IL4, as described in Methods. *$P < 0.05$, **$P < 0.01$ vs. control. Each bar represents mean ± SD ($n = 3$).

increase, in a time-dependent manner, the adherence of THP-1 cells to plastic surfaces. Although, PMA- and PMA + IL-4-treated THP-1 monocytes showed higher adhesive ability than sPLA$_2$-IIA-treated cells, after 48 h the adherence induced by both agents showed similar levels of increase than at 24 h.

4. Discussion

Inflammation plays an important role in atherosclerosis, contributing to the recruitment of inflammatory cells into the lesions, where they turn into macrophages, foam cells, or dendritic cells, causing plaque instability, which is the main process that elicits cardiovascular events. It is well demonstrated that cardiovascular risk factors, such as oxidized-LDL, nicotine, or some acute-phase reactants (APR), cause the modulation of the immune system.[28–30] Among APR found enhanced in serum of patients with coronary artery disease, high circulating levels of C-reactive protein and sPLA$_2$-IIA have been observed, but, although C-reactive protein role in DC differentiation, maturation, and functions have been assessed, little is known about how secreted phospholipases affect this process.[30]

Several attempts have been performed with bee venom PLA$_2$ and the catalytic domain of human sPLA$_2$-III on DC maturation and activation,[12,31] but the role in immunity and/or in the tolerance of the sPLA$_2$ enzymes present in human inflammatory lesions, such as sPLA$_2$-IIA, sPLA$_2$-V, or sPLA$_2$-X, still has to be addressed. This work is the first to evaluate the potential immunomodulatory effects of the human sPLA$_2$-IIA. In this study, human THP-1 monocytes, widely used as a monocyte/macrophage differentiation model, were characterized following incubation with the secreted enzyme. We show that, upon treatment, THP-1 cells display the following: (i) upregulation of surface markers CD83, CD40, DC-SIGN, CD54, CD61, and CD62L; (ii) downregulation of surface marker CD14; (iii) appearance of typical dendritic morphological features; (iv) adhesion and migration; (v) increased endocytic activity; and (vi) induction of allogeneic lymphocyte proliferation. Therefore, we conclude that sPLA$_2$-IIA, an enzyme present in the atherosclerotic lesions, is able to induce the differentiation of THP-1 monocytes into MDDCs or, alternatively activated macrophages, similar to what is observed with the classical combination of PMA + IL-4.[25] Moreover, the studies that we performed in human monocytes to validate these responses confirmed that the differentiation activity of the phospholipase is not restricted to THP-1 cells.

Previously, it had been suggested that the participation of inflammatory-secreted PLA$_2$s, including group IIA, V, or X, on DC maturation *in vivo*, is due to their ability to modify LDL, rather than phospholipases themselves.[32,33] However, here we show that it is the action of sPLA$_2$-IIA itself on THP-1 monocytes which promotes the phenotypic and morphological modifications.

In atherosclerotic arteries, sPLA$_2$-IIA occurs in both cellular and extracellular elements, and it has been found associated to SMC and macrophages/foam cells.[20] Therefore, we can hypothesize that sPLA$_2$-IIA released by SMC may induce a dendritic cell-like phenotype in adjacent monocytes/macrophages, acting as an amplifier of the inflammatory responses and enabling these cells to present antigens and support an immune response.

$sPLA_2$-IIA-treated THP-1 cells are, in many ways, comparable with *in vitro*-differentiated monocytes in the presence of PMA + IL-4. They become clustered and develop the clearly characteristic dendritic morphology, as well as the capacity to capture FITC-dextran. However, although they share the basic DC phenotype, the analysis of the expressed membrane markers demonstrates some differences between them, for instance, although after PMA + IL-4 differentiation cells acquire high expression levels of CD11b, CD11c, CD1a, and CD18, $sPLA_2$-IIA treatment does not affect their expression.

It has been suggested that depending on the local microenvironment, MDDC can adopt different membrane-marker profiles, which could have major influences on cell–cell interactions and antigen uptake and presentation. Thus, the absence of CD1a expression has also been found in DCs derived from human monocytes in the presence of dexamethasone.[34] In addition, and consistent with the role of CD1a in the allogenic T cell response, dexamethasone DC has lower T cell stimulatory activity. In keeping with that, although we have observed that $sPLA_2$-treated THP-1 cells present the ability to induce higher lymphocyte proliferation than resting ones, their allostimulatory function is weaker than that induced by PMA + IL4 differentiation, which also elicits CD1a upregulation.

Another crucial function of DCs is the capacity to capture exogenous antigens: the endocytic activity. We found that $sPLA_2$-IIA significantly altered the processes of endocytosis and the expression of some phagocytic receptors on THP-1 cells. The endocytic capacity of $sPLA_2$-IIA-treated cells was significantly enhanced, although at a lower extent than PMA + IL-4-differentiated cells. However, the expression of DC-specific ICAM-3-grabbing non-integrin was largely upregulated in both conditions. This increase on surface DC-SIGN expression presents a special relevance, given that studies on plaques from human coronary and carotid arteries and aorta reveal the presence of DC-SIGN-immunoreactive cells.[10] In addition, since this DC-specific lectin molecule has been implicated as a receptor used by various microorganisms associated with chronic infection, its upregulation on $sPLA_2$-IIA-treated cells may also have important consequences for pathogen clearance. Thus, THP-1 differentiation in the presence of $sPLA_2$-IIA at the sites of inflammation may directly play a role in antigen loading, presentation capabilities, and, therefore, in the immune response. Interestingly, we (and others) detect at least three DC-SIGN bands in differentiated cells; it has been suggested that they may represent differential glycosylation of a DC-SIGN isoform or be derived from alternatively spliced mRNA but it is still under investigation.[25]

In addition, the observed upregulation of CD83, a typical marker of DCs, was also recently reported for monocyte-derived DCs treated with $sPLA_2$-III from bee venom.[12] The adhesion molecules, CD54 (involved in leucocyte trafficking towards inflammatory stimuli) and CD62L (the homing receptor), are also increased during the time-course analysis, although CD18, also required for migration, is not modulated. The expression of co-stimulatory molecules, such as CD40, also increased following 24 h of phospholipase stimulation. Therefore, the expression marker profile induced by $sPLA_2$-IIA resembles an intermediate stage between immature and mature MDDC.

It is generally accepted that many types of DCs exist, with subtle differences in phenotype markers, and there is a wide array of factors that regulate monocyte differentiation, playing a critical role on the heterogenous phenotype of DC. Thus, depending on the presence of specific cytokines, monocyte differentiation switches from dendritic cells to macrophages, or from macrophages to DC.[35,36] Factors, such as cyclic nucleotides, promote monocyte differentiation towards an intermediate cell type between macrophages or DCs.[37] Even foam cell-inducing agents, such as native LDL and oxLDL, promote a dendritic cell-like phenotype on macrophages.[38]

In conclusion, this work shows that the immunostimulatory activity of secreted phospholipases is not restricted to the induction of DC maturation, but can also be considered environmental factors, at the inflammatory site, that affect the differentiation process of monocytes, playing a crucial role in the interface among innate and adaptive immunity.

Increased PLA_2 levels in atherosclerotic arteries may be a critical factor to regulate dendritic cell formation in lesions favouring the development of the adaptive arm of immunity and, in turn, influencing composition, inflammatory nature, and plaque growth. The regulation of the $sPLA_2$ expression in the atherosclerotic lesion could be a strategy in order to avoid plaque instability and, therefore, a worsening of the prognosis of the pathology.

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