Secreted phospholipase A₂ type IIA as a mediator connecting innate and adaptive immunity: new role in atherosclerosis

Elvira Ibeas[†], Lucía Fuentes[†], Rubén Martín, Marita Hernández, and Maria Luisa Nieto*

Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas/Universidad de Valladolid, C/ Sanz y Fores s/n., 47005 Valladolid, Spain

Received 3 December 2007; revised 29 July 2008; accepted 22 August 2008; online publish-ahead-of-print 28 August 2008

Time for primary review: 23 days

KEYWORDS

Atherosclerosis;
Dendritic cells;
Macrophages;
Inflammation;
sPLA₂-IIA

Aims Human atherosclerotic plaques express markers of macrophage/dendritic cells as well as high levels of inflammatory proteins such as secreted phospholipase A₂ type IIA (sPLA₂-IIA). To understand the cellular changes associated with the progress of atherosclerosis, we evaluated the role of sPLA₂-IIA in mediating monocyte recruitment and differentiation into antigen-presenting cells.

Methods and results The effect of sPLA₂-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. sPLA₂-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, sPLA₂-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 sPLA₂-IIA treatment.

Conclusion sPLA₂-IIA induces the differentiation of mononuclear cells and increases their adhesive and migratory capabilities, which suggests a novel function for sPLA₂-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 T cell-mediated immunity.^{11–13}

A multitude of signals, including bacterial components such as LPS, or pro-inflammatory factors such as TNF α or IL-1 β , 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 A₂ type III (sPLA₂-III), have indicated that sPLA₂-III can also act on DC

* Corresponding author. Tel: +34 983 184836; fax: +34 983 184800.
E-mail address: mlnieto@ibgm.uva.es

[†] These two authors contributed equally to this work.

membranes mobilizing lipid mediators and inducing their maturation.¹⁵

Mammalian-secreted PLA₂s 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 Ca²⁺ for enzymatic activity.¹⁶ The plasma of patients with various inflammatory diseases, particularly atherosclerosis, contains high concentrations of the isoform sPLA₂-IIA, and clinical studies reveal a correlation between circulating levels of the phospholipase and vascular complications.¹⁷

sPLA₂-IIA, which can be upregulated by inflammatory cytokines, such as IL-1 β , IL-6, and TNF α , 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. sPLA₂-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 sPLA₂-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 sPLA₂-IIA in monocyte biology and we found that sPLA₂-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 sPLA₂-IIA from human placenta was used as a source of human recombinant enzyme. sPLA₂-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 TNF α and IL-4 were from Calbiochem. Monoclonal anti-human DC-SIGN was a generous gift from Dr A.L. Corbí (Centro de Investigaciones Biológicas, CSIC, Madrid, Spain).

2.2 Cell culture

THP-1 cells were cultured at 37°C under 5% CO₂ 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 μ g/mL sPLA₂-IIA or 0.2 μ M 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 μ g/mL sPLA₂-IIA, 0.2 μ M of PMA, or 0.2 μ M 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 (Pharmin-gen, San Diego, CA, USA). Control and treated cells were collected for immunolabelling with 10 μ g/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 μ L 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 sPLA₂-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-³H]-thymidine-labelled adhesion assay. Briefly, THP-1 cells (5×10^6) were incubated in RPMI/5% FBS in the presence of [methyl-³H]-thymidine (Amersham, UK) at a final concentration of 0.5 μ Ci/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 μ g/mL of sPLA₂-IIA, 0.2 μ M of PMA, or 0.2 μ M 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 μ m 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 μ g/mL of sPLA₂ 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 sPLA₂-IIA-treated THP-1 cells to activate T cells was measured in an MLR and quantified by the MTS assay (Cell Titer 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 sPLA₂-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 (10³, 5 × 10³, 10⁴) of these differentiated THP-1 cells (THP-1-DC) were added to freshly isolated 10⁴ 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 A₂ type IIA promotes THP-1 monocyte differentiation

We studied the capacity of sPLA₂-IIA to modulate monocyte differentiation into either macrophages or DCs. In order to

Figure 1 Secreted phospholipase A₂ type IIA (sPLA₂-IIA) reduces the expression of CD14 on THP-1 cells. Cells were treated with 1 µg/mL of sPLA₂-IIA for various time periods at 37°C (A) or for 48 h with different doses of sPLA₂-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 sPLA₂-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 sPLA₂-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 sPLA₂-IIA triggered a time-dependent downregulatory effect on CD14 surface expression. To define the dose-dependency of the action of the sPLA₂-IIA, cells were incubated for 24 h with different concentrations of sPLA₂-IIA. *Figure 1B* shows the effects of the different concentrations of sPLA₂-IIA on CD14 downregulation. The optimal effect took place at a concentration of 1 µg/mL sPLA₂-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 sPLA₂-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 sPLA₂-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.

The effect found on CD14 surface expression after sPLA₂-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 sPLA₂-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, sPLA₂-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 sPLA₂-IIA or PMA and IL-4 compared with untreated cells.

To go deeper into the phenotype of sPLA₂-IIA-treated THP-1 monocytes, flow-cytometrical analysis of monocyte/dendritic surface antigens was performed (*Figure 4*). The histograms demonstrate that after sPLA₂-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 sPLA₂-IIA (data not shown).

Figure 2 Secreted phospholipase A₂ type IIA (sPLA₂-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 A₂ 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.

Figure 3 Secreted phospholipase A₂ type IIA (sPLA₂-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 A₂ 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 cells 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

3.2 Effects of secreted phospholipase A₂ type IIA on functional cellular processes

3.2.1 Regulation of endocytosis by secreted phospholipase A₂ 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 sPLA₂-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 sPLA₂-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 sPLA₂-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 sPLA₂-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 10⁴ 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 sPLA₂-IIA- or PMA + IL4-stimulated THP-1 cells, respectively, at the same ratio. Therefore, sPLA₂-IIA generates cells that possess the capacity to trigger functionally relevant interactions with T lymphocytes.

3.2.3 Regulation of chemotaxis by secreted phospholipase A₂ type IIA

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

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 A₂ 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).

Figure 4 Phenotype of secreted phospholipase A₂ type IIA (sPLA₂-IIA)-treated cells. THP-1 cells were cultured in the presence of 1 μ g/mL secreted phospholipase A₂ type IIA or 0.2 μ M 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 TNF α .²⁷ To determine the migratory capacity of the cells in response to sPLA₂-IIA or to sPLA₂-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 sPLA₂-IIA-stimulated cells, or 24 h TNF α -stimulated cells. With the 8 μ m pore inserts, THP-1 cells showed a slight preferential migration in response to supernatants from sPLA₂-IIA-stimulated cells, over TNF α -supernatants, and migrated three-fold above the media

Figure 5 Endocytic capacity of secreted phospholipase A₂ type IIA (sPLA₂-IIA)-treated cells. THP-1 cells (A and B) and human monocytes (C and D) were cultured in the absence or presence of 1 μ g/mL secreted phospholipase A₂ type IIA, 0.2 μ M PMA, or 0.2 μ M 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 $\pm$ SD normalized by setting controls to 100%. * P < 0.05, ** P < 0.01 vs. control untreated cells.

control (Figure 6B). With the 3 μ m pore inserts, the migration of THP-1 cells exposed to sPLA₂-IIA supernatant was sensibly diminished, whereas those exposed to TNF α supernatants showed similar levels of chemotaxis to the 8 μ m pore inserts. When the lower chambers contained media plus sPLA₂-IIA, no chemotactic effect was observed. In addition, we examined the migration of sPLA₂-IIA-treated cells exposed to sPLA₂-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 A₂ 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 sPLA₂-IIA to affect cell adherence, [³H]-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, sPLA₂-IIA, as well as PMA or PMA + IL-4, was found to

Figure 6 Effect of secreted phospholipase A₂ 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 A₂ type IIA or 0.2 μM PMA + 1000 U/mL IL4 for 5 days and treated with mitomycin C. Then, 10⁴ 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 A₂ 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 sPLA₂-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 sPLA₂-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 PLA₂ and the catalytic domain of human sPLA₂-III on DC maturation and activation,^{12,31} but the role in immunity and/or in the tolerance of the sPLA₂ enzymes present in human inflammatory lesions, such as sPLA₂-IIA, sPLA₂-V, or sPLA₂-X, still has to be addressed. This work is the first to evaluate the potential immunomodulatory effects of the human sPLA₂-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 sPLA₂-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 PLA₂s, 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 sPLA₂-IIA itself on THP-1 monocytes which promotes the phenotypic and morphological modifications.

In atherosclerotic arteries, sPLA₂-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 sPLA₂-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.

sPLA₂-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, sPLA₂-IIA treatment does not affect their expression.

It has been suggested that depending on the local micro-environment, MDSC 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 sPLA₂-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 sPLA₂-IIA significantly altered the processes of endocytosis and the expression of some phagocytic receptors on THP-1 cells. The endocytic capacity of sPLA₂-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 sPLA₂-IIA-treated cells may also have important consequences for pathogen clearance. Thus, THP-1 differentiation in the presence of sPLA₂-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 sPLA₂-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 sPLA₂-IIA resembles an intermediate stage between immature and mature MDSC.

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 PLA₂ 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 sPLA₂ 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.

Funding

This work was supported by Plan Nacional de Salud y Farmacia (SAF2005-01242) and Junta de Castilla y Leon (CSI11A08). M.H. is under the Ramon y Cajal Program (co-funded by F.S.E.). E.I. and R.M. are supported by a fellowship from the Spanish Ministerio de Educación y Ciencia.

Acknowledgements

We thank P. Maeso and M. García (supported by the Red de Centros RECAVA) for their expert technical support, and we are grateful to M. Balboa and Y. Saez for providing human monocytes and lymphocytes, and to D. Bernardo for the assistance with MLR assay.

Conflict of interest: none declared.

References

1. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med* 1999;**340**: 115–126.
2. Wick G, Schett G, Amberger A, Kleindeinst R, Xu Q. Is atherosclerosis an immunologically mediated disease? *Immunology Today* 1995;**16**:27–33.
3. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* 2002;**91**:281–291.
4. Nicoletti A, Caligiuri G, Hansson GK. Immunomodulation of atherosclerosis: myth and reality. *J Intern Med* 2000;**247**:397–405.
5. Link A, Bohm M. Potential role of dendritic cells in atherosclerosis. *Cardiovasc Res* 2002;**55**:708–709.
6. Lord RS, Bobryshev YV. Clustering of dendritic cells in athero-prone areas of the aorta. *Atherosclerosis* 1999;**146**:197–198.
7. Millong G, Malcom GT, Wick G. Early inflammatory-immunological lesions in juvenile atherosclerosis from The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study. *Atherosclerosis* 2002;**160**:441–448.
8. Millonig G, Niederegger H, Rabl W, Hochleitner BW, Hofer D, Romani N et al. Network of vascular-associated dendritic cells in intima of healthy young individuals. *Arterioscler Thromb Vasc Biol* 2001;**21**: 503–508.

9. Ozem J, Bobryshev YV, Lord RS, Ashwell KW. Identification of dendritic cells in aortic atherosclerotic lesions in rats with diet-induced hypercholesterolemia. *Histol Histopathol* 2002;17:223–237.
10. Soilleux EJ, Morris LS, Trowsdale J, Coleman N, Boyle JJ. Human atherosclerotic plaques express DC-SIGN, a novel protein found on dendritic cells and macrophages. *J Pathol* 2002;198:511–516.
11. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA *et al*. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4⁺ T cells. *J Immunol* 1995;154:5071–5079.
12. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003;21:685–711.
13. Heath WR, Belz GT, Behrens GM, Smith CM, Forehan SP, Parish IA *et al*. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev* 2004;199:9–26.
14. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ *et al*. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;18:767–811.
15. Ramoner R, Putz T, Gander H, Rahm A, Bartsch G, Schaber C *et al*. Dendritic-cell activation by secretory phospholipase A₂. *Blood* 2005;105:3583–3587.
16. Six DA, Dennis EA. The expanding superfamily of phospholipase A₂ enzymes: classification and characterization. *Biochim Biophys Acta* 2000;1488:1–19.
17. Liu PY, Li YH, Tsai WC, Chao TH, Tsai LM, Wu HL *et al*. Prognostic value and the changes of plasma levels of secretory type II phospholipase A₂ in patients with coronary artery disease undergoing percutaneous coronary intervention. *Eur Heart J* 2003;24:1824–1832.
18. Andreani M, Olivier J-L, Berenbaum F, Raymondjean M, Bérézat G. Transcriptional regulation of inflammatory secreted phospholipases A₂. *Biochim Biophys Acta* 2000;1488:149–158.
19. Menschikowski M, Kasper M, Lattke P, Schiering A, Schiefer S, Stockinger H *et al*. Secretory group II phospholipase A₂ in human atherosclerotic plaques. *Atherosclerosis* 1995;118:173–181.
20. Camejo EH, Andersen S, Standal R, Rosengren B, Sartipy P, Stadberg E *et al*. Localization of secretory non-pancreatic phospholipase A₂ in normal and atherosclerotic arteries: activity of the isolated enzyme on low density lipoproteins. *Arterioscler Thromb Vasc Biol* 1997;17:300–309.
21. Hakala JK, Öörni K, Pentikäinen MO, Eva Hurt-Camejo E, Kovanen PT. Lipolysis of LDL by human secretory phospholipase A₂ induces particle fusion and enhances the retention of LDL to human aortic proteoglycans. *Arterioscler Thromb Vasc Biol* 2001;21:1053–1058.
22. Tada K, Murakami M, Kambe T, Kudo I. Induction of cyclooxygenase-2 by secretory phospholipases A₂ in nerve growth factor-stimulated rat serosal mast cells is facilitated by interaction with fibroblasts and mediated by a mechanism independent of their enzymatic functions. *J Immunol* 1998;161:5008–5015.
23. Hernandez M, Fuentes L, Fernandez-Aviles F, Crespo MS, Nieto ML. Secretory phospholipase A₂ elicits proinflammatory changes and upregulates the surface expression of Fas ligand in monocytic cells: potential relevance for atherogenesis. *Circ Res* 2002;90:38–45.
24. Jaulmes A, Janvier B, Andrean M, Raymondjean M. Autocrine and paracrine transcriptional regulation of type IIA secretory phospholipase A₂ gene in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2005;25:1161–1167.
25. Puig-Kröger A, Serrano-Gómez D, Caparrós E, Domínguez-Soto A, Rellosio M, Colmenares M *et al*. Regulated expression of the pathogen receptor dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin in THP-1 human leukemic cells, monocytes, and macrophages. *J Biol Chem* 2004;279:25680–25688.
26. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 2001;106:255–258.
27. Roake JA, Rao AS, Morris PJ, Larsen CP, Hankins DF, Austyn JM. Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J Exp Med* 1995;181:2237–2247.
28. Alderman CJ, Bunyard PR, Chain BM, Foreman JC, Leake DS, Katz DR. Effects of oxidized low density lipoprotein on dendritic cells: a possible immunoregulatory component of the atherogenic microenvironment? *Cardiovascular Res* 2002;55:806–819.
29. Aicher AH, Heeschen C, Mohaupt M, Cooke JP, Zeiher AM, Dimmeler S. Nicotine strongly activates DC-mediated adaptive immunity: potential role for progression of atherosclerotic lesions. *Circulation* 2003;107:604–611.
30. Zhang R, Becnel L, Li M, Chen C, Yao Q. C-reactive protein impairs human CD14⁺ monocyte-derived dendritic cell differentiation, maturation and function. *Eur J Immunol* 2006;36:2993–3006.
31. Perrin-Cocon L, Agaue S, Coutant F, Masurel A, Bezzine S, Lambeau G *et al*. Secretory phospholipase A₂ induces dendritic cell maturation. *Eur J Immunol* 2004;34:2293–2302.
32. Coutant F, Agaue S, Perrin-Cocon L, André P, Lotteau V. Sensing environmental lipids by dendritic cell modulates its function. *J Immunol* 2004;172:54–60.
33. Coutant F, Perrin-Cocon L, Agaue S, Delair T, André P, Lotteau V. Mature dendritic cell generation promoted by lysophosphatidylcholine. *J Immunol* 2002;169:1688–1695.
34. Xia C-Q, Peng R, Beato F, Clare-Salzler MJ. Dexamethasone induces IL-10-producing monocyte-derived dendritic cells with durable immaturity. *Scandinavian J Immunol* 2005;62:45–54.
35. Delneste Y, Charbonnier P, Herbault N, Magistrelli G, Caron G, Bonnefoy J-Y *et al*. Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages. *Blood* 2003;101:143–150.
36. Chomarat P, Dantin C, Bennett L, Banchereau J, Palucka AK. TNF skews monocyte differentiation from macrophages to dendritic cells. *J Immunol* 2003;171:2262–2269.
37. Giordano D, Magaletti DM, Clark EA, Beavo JA. Cyclic nucleotides promote monocyte differentiation toward a DC-SIGN⁺ (CD209) intermediate cell and impair differentiation into dendritic cells. *J Immunol* 2003;171:6421–6430.
38. Cho HJ, Shashkin P, Gleissner CA, Dunson D, Jain N, Lee JK *et al*. Induction of dendritic cell-like phenotype in macrophages during foam cell formation. *Physiol Genomics* 2007;29:149–116.