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Secretory phospholipase A₂ induces dendritic cell maturation

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Abbreviations: **APR:** acute phase response; **LDL:** low density lipoprotein; **LPC:** lyso-phosphatidylcholine; **LPDS:** lipoprotein deficient serum; **oxLDL:** oxidized low density lipoprotein; **PAF:** platelet-activating factor; **PLA₂:** phospholipase A₂; **PC:** phosphatidylcholine; **PPAR:** peroxisome proliferator-activated receptor; **sPLA₂:** secreted PLA₂, **hGIII sPLA₂:** human group III sPLA₂.

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Abstract

High level of phospholipase A₂ (PLA₂) activity is found in serum and biological fluids during the acute phase response (APR). Extracellular PLA₂ in fluids of patients with inflammatory diseases such as sepsis, acute pancreatitis or rheumatoid arthritis is also associated with propagation of inflammation. PLA₂ activity is involved in the release of both pro- and anti-inflammatory lipid mediators from phospholipids of cellular membranes or circulating lipoproteins. PLA₂ may thus generate signals that influence immune responses. Here, group III secretory PLA₂s were tested for their ability to promote generation of functionally mature human dendritic cells (DC). PLA₂ treatment of differentiating monocytes in the presence of GM-CSF and IL-4 yielded cells with phenotypical and functional characteristics of mature DC. This maturation was dependent on the dose of PLA₂ and PLA₂-generated DC stimulated interferon gamma secretion by allogeneic T cells. The effects of PLA₂ on DC maturation was mainly dependent on enzyme activity and correlated with the activation of NF- κ B, AP-1 and NFAT. The data suggest that transient increase in PLA₂ activity generates signals that promote transition of innate to adaptive immunity during the APR.

1 Introduction

The acute phase response (APR) is a non specific physiological response to aggression of the organism whose main function is to favor tissue repair and pathogen elimination. This alarm system can be triggered by different kinds of stress such as injury, physical trauma or infection and result in the release of inflammatory mediators that induce transient and drastic modifications of plasma composition [1]. The concentration of numerous plasma proteins called acute phase reactants can be increased up to a thousand fold while that of other proteins like albumin is drastically decreased [2]. This systemic response of APR has numerous consequences especially on lipid metabolism. It is accompanied by alterations in lipoprotein composition and result in accumulation of oxidized low density lipoproteins (LDL) and generation of modified phospholipids [3, 4]. Because phospholipase A₂ (PLA₂) activity increases following cell injury and during inflammatory conditions, secretory PLA₂ can be considered as an acute phase reactant [5].

PLA₂s are enzymes that hydrolyze phospholipids at the sn-2 position to produce lysophospholipids and free fatty acids. Mammalian PLA₂ isoenzymes are subdivided into four families, including cytosolic calcium-dependent and independent PLA₂s, secretory PLA₂s (sPLA₂) and PAF-acetylhydrolases [6]. sPLA₂s are low molecular weight enzymes that use calcium as cofactor. High amounts of sPLA₂s are also found in snake and bee venoms. Bee venom group III sPLA₂ is the major allergen of hymenoptera venom and is largely used in desensitization treatments [7]. A human group III sPLA₂ (hGIII sPLA₂) with high homology to the bee venom enzyme has recently been described [8]. High level of sPLA₂ has been measured in synovial fluid of rheumatoid arthritis patients [9, 10], bronchoalveolar lavage of asthmatic patients [11] or in serum of individuals with systemic inflammatory disorders such

as septic shock [12], acute pancreatitis [13] and autoimmune diseases [14]. Some sPLA₂s were characterized as acute phase reactants playing a role in atherosclerosis by altering high and low density lipoprotein catabolism and uptake [15].

The effects of sPLA₂s can be associated to their catalytic properties or to the engagement of cellular receptors [16, 17]. Different types of membrane receptors for sPLA₂s have been identified. N-type receptors are mostly expressed in brain and bind neurotoxic sPLA₂ with high affinity. M-type receptors that belong to the C-type lectin family are expressed in various tissues and display low affinity for the bee venom PLA₂ (for review see [18]). Binding of PLA₂ to the mannose receptor and glycosaminoglycans has also been reported and may trigger cellular activation and release of bioactive molecules like cytokines, leukotriene B₄ and platelet-activating factor (PAF) [19-21].

PLA₂ hydrolysis of PC can generate bioactive lipids such as lysophosphatidylcholine (LPC) and non esterified poly-unsaturated fatty acids that exert various effects on immune cells. We have recently shown that LPC promotes mature DC generation from differentiating monocytes [22, 23]. Non esterified poly-unsaturated fatty acids can modulate toll-like receptor (TLR) signaling, resulting in modified activation of NF- κ B [24]. Furthermore, eicosanoids which are downstream metabolites of the PLA₂ reaction, are involved in the regulation of functional properties of dendritic cells (DCs) with diverse effects. For instance, lipoxin A₄ strongly inhibits IL-12 production by mature DCs [25], and prostaglandin E₂ regulates the migratory capacity of DCs [26] and their secretion of IL-12 [27].

PLA₂-mediated processing of PC can generate a number of bioactive molecules with opposite effects on immunity. Since the secretion of PLA₂s is strongly stimulated during the acute phase response, we asked whether an increased PLA₂ activity can generate signals identified by the innate immune system that would promote transition to an adaptive response. Here we

describe the effect of group III sPLA₂s on the generation of functional human DC derived from monocytes.

2 Results

2.1 Phenotypic maturation induced by group III sPLA₂

We have previously shown that LPC can induce the differentiation of mature DC directly from monocytes when it is added for the last 24 h of monocyte differentiation in the presence of GM-CSF and IL-4 [22, 23]. LPC can be generated from PC by PLA₂ hydrolysis. Therefore, we asked whether sPLA₂ could exert the same effects on differentiating monocytes. As expected, monocytes incubated for 6 days with GM-CSF and IL-4 differentiated to immature DC (control) expressing high levels of CD1a but no CD14, low levels of HLA-DR and CD40 but no CD86, CD80 and CD83 (figure 1A). When bee venom sPLA₂ was introduced in the culture medium for the last 24 h of differentiation, cells obtained at day 6 had the phenotype of mature DC with enhanced expression of HLA-DR, CD86, CD80, CD83 and CD40. The maturation induced by this PLA₂ was weaker than that induced by LPS (figure 1A), especially for the up-regulation of CD83 and CD40. The addition of the endotoxin inhibitor polymixin B had no effect on PLA₂-induced maturation (figure 1A) whereas it strongly inhibited an important dose of LPS (10 ng/ml) (data not shown). The maturation was dependent on the dose of PLA₂ and maximal CD86 induction was obtained with 5 U/ml of PLA₂ (figure 1B). Further experiments with bee venom PLA₂ were therefore realized with 5 U/ml.

2.2 Functional mature DC promoted by group III sPLA₂

To analyze their functional properties, PLA₂-treated DC were washed and co-cultured with allogeneic T cells. We found that these DC were able to stimulate IFN γ secretion (figure 2A) whereas no IL-4 nor IL-5 secretion could be detected (data not shown). The concomitant

addition of polymixin B with PLA₂ during DC differentiation did not affect the functional maturation induced by PLA₂ (data not shown). To confirm the Th1 polarization of T cells, intracellular IL-4 and IFN γ synthesis by T cells was analyzed after restimulation by IL-2. T cells co-cultured with immature control DC led to a low number of IFN γ -producing cells and no production of IL-4 (figure 2B). Although with less efficiency than cells treated with 1 μ g/ml LPS, PLA₂-generated DC increased the percentage of IFN γ -secreting T cells but not that of IL-4-producing T cells (figure 2B). Therefore, bee venom PLA₂ was able to generate mature DC that have the ability to induce a Th1-oriented response in vitro. This polarization could not be explained by IL-12 secretion, since PLA₂-treated cells did not secrete IL-12p70 and secreted small amounts of IL-12p40 (43 ± 29 pg/ml) that were only slightly above that of untreated cells (13 ± 9 pg/ml).

2.3 The catalytic domain of human group III sPLA₂ induces DC maturation

The catalytic domain of the human homologue of group III bee-venom PLA₂ (hGIII sPLA₂), that was produced as a recombinant protein in S2 insect cells and purified to homogeneity [28] also induced the maturation of DC, enhancing the expression of presenting and costimulatory molecules (figure 3A). These cells were functionally mature since they also stimulated the secretion of IFN γ by allogeneic T cells (figure 3B). Because of the rarity of this purified human PLA₂, the following experiments were performed with the bee venom enzyme.

2.4 PLA₂ binding and internalization by DCs

Bee venom PLA₂ was labeled with fluorescent Alexa488 to follow its binding and internalization by cells. Differentiating monocytes were harvested at day 5 (before any treatment by PLA₂) and incubated on ice with increasing doses of fluorescent PLA₂. The binding of PLA₂ on cells was low, especially at the dose of 4 µg/ml, corresponding to the optimal active dose of 5 U/ml. Nevertheless, PLA₂ binding reached saturation at 8 µg/ml (~500 nM) for 10⁶ cells (figure 4A). This binding could be inhibited by competition with non-labeled PLA₂ and 80 % inhibition was observed with a ten-fold excess of non labeled PLA₂ (figure 4B). The data suggest that PLA₂ binds to a specific receptor. The high concentration of bee venom PLA₂ (~500 nM) necessary to reach saturation suggests that this receptor may be of low affinity. Chelation of calcium by 2 mM EDTA and addition of 5 µg/ml of mannan inhibited the binding of Alexa-PLA₂ by 50 % and inhibitions by mannan and EDTA were not additive (data not shown), suggesting that at least one C-type lectin is involved in PLA₂ binding to differentiating monocytes. These results may be in line with those of Mukhopadhyay and Stahl showing that bee venom PLA₂ binds to the macrophage mannose receptor (CD206) [29], a C-type lectin that is expressed on immature DCs.

When the enzyme was incubated with cells at 37°C, the fluorescent PLA₂ was efficiently and rapidly internalized (figure 4C). Internalized PLA₂ could be visualized by a punctuated labeling in almost all cells (figure 4D). Binding / Internalization experiments revealed that the membrane receptor for PLA₂ could trigger internalization of its ligand (figure 4E). Therefore, during the 24 h treatment of DC, PLA₂ is likely to be internalized by both receptor-mediated endocytosis and constitutive pinocytosis which is highly efficient in immature DCs. The relative contribution of each pathway remains to be determined.

2.5 Mechanism of action of sPLA₂

The effect of PLA₂ on DCs may be due either to a direct enzymatic effect or to membrane signals induced by PLA₂ binding to surface receptors. The catalytic activity of the enzyme can be inactivated by progressive heating at 95°C, as measured by hydrolysis of arachidonoyl-thio-PC (figure 5A). After denaturation of PLA₂ for various periods of time (0 to 30 min), the same amount of enzyme (4 µg/ml) was added to the culture medium at day 5 of monocyte differentiation. The maturation state of the cells was estimated 24 hours later by analyzing their phenotype. We previously observed that CD86 is the most sensitive and reliable marker of activation, therefore CD86 expression was plotted according to the enzyme activity measured after heating (figure 5B). The induction of maturation correlated with the phospholipase activity, with a drastic decrease in CD86 induction when PLA₂ was heated for only 5 min. After heat-inactivation of the enzyme for 30 min, its binding to DCs was not affected (figure 5C), indicating that sPLA₂ binding is not involved or not sufficient by itself to induce DC activation. Thus, the generation of mature DC by PLA₂ appears to depend on the enzyme activity.

2.6 Transcription factors activated by sPLA₂

Activation signals of DC can induce maturation through several distinct pathways. Signals like those engaging Toll-Like Receptors (TLR) or inflammatory cytokine receptors result in the activation of NF-κB. Others like DC maturation triggered by FcR or LPC are independent of NF-κB. The role of nuclear factors was studied by electrophoretic mobility shift assays (EMSA) using specific DNA probes. As shown in figure 6 (A and C), NF-κB is activated by PLA₂ treatment. NF-κB binding activity is induced after 4 hours of treatment with PLA₂ and remains highly activated after 24 hours. PLA₂ treatment also induced the activation of AP-1 and NFAT (figure 6B-C). Peroxisome proliferator-activated receptors gamma (PPAR_γ) are

ligand-dependent transcription factors that are inhibited by LPC and activated by lipids such as HODE and HETE derived from linoleic and arachidonic acid [30]. They are involved in the regulation of inflammation and lipid metabolism. Using a DNA probe containing a peroxisome proliferator response element from CD36 promoter, no significant variation of PPAR γ could be observed (figure 6B-C).

3 Discussion

The aim of this work was to determine whether an increase in sPLA₂ activity resulted in the release of endogenous lipid signals that can be detected by the innate immune system. In this paper, it is shown that treatment of differentiating monocytes by the bee venom PLA₂ and the catalytic domain of hGIII sPLA₂ generates cells with phenotypic and functional characteristics of mature DCs. PLA₂-generated DC could activate a Th1 type response by allogeneic T cells, inducing the secretion of IFN γ but not that of IL-4. Although the bee venom PLA₂ binds to specific membrane receptors on DCs, its biological effects seem to be associated to its enzymatic activity. We could exclude PLA₂ contamination by endotoxins because: *i*, polymixin B did not affect PLA₂-induced phenotypic and functional maturation, *ii*, sPLA₂ did not induce important secretion of IL-12 and *iii*, heating of the enzyme completely abolished its effect. PLA₂ treatment leading to mature DC generation also activated transcriptional factors known to be involved in the activation and regulation of an immune response. The data supported and extended the notion recently proposed that lipid mediators, whose production is highly controlled during the APR, may play a central role in immunoregulation.

The biological effect of the bee venom PLA₂ on DCs appears dependent on its enzymatic activity. Although most of the study was performed with bee-venom PLA₂, similar data were obtained with the highly purified catalytic domain of hGIII sPLA₂. Several other PLA₂ may also participate to DC maturation *in vivo*. For instance, the lipoprotein-associated plasmatic PLA₂ also called PAF-acetylhydrolase hydrolyzes oxidized phospholipids with shortened fatty acid in sn-2 position, increasing LPC content in oxLDL [31]. The human group IIA, V or X sPLA₂s produced under inflammatory conditions can also modify lipoproteins and generate high amounts of LPC in LDL [32-34]. Lipolytic modifications of LDL by bee venom PLA₂

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increased the affinity of LDL for proteoglycans and glycosaminoglycans [35]. Under our experimental conditions, addition of purified LDL in the culture medium did not enhance PLA₂ effect on DC generation (data not shown). This may be due to the presence of albumin which is known to retain and inactivate fatty acids and lysophospholipids generated by PLA₂ hydrolysis of native lipoproteins. Therefore these lipid mediators are likely to be the most efficient when generated via PLA₂-mediated processing of membrane phospholipids. PLA₂ could also hydrolyze phospholipids from membranes of endocytic compartments following internalization of the enzyme. Human group II, V, X and XII as well as human and bee-venom group III sPLA₂ have been reported to generate external lipid mediators and hydrolyze phospholipids from the outer membrane leaflet [28, 36]. Lipids generated at the cell membrane may be directly detected by membrane receptors or enter the cells to be metabolized or directly interact with nuclear receptors.

The treatment of DCs with the bee venom PLA₂ results in NF- κ B, AP-1 and NFAT activation. These transcription factors control the expression of numerous genes involved in the regulation of immunity. The role of NF- κ B in DC maturation has been extensively studied [37-39]. This nuclear factor regulates genes involved in inflammation such as inflammatory cytokines (IL-1 β , TNF α , IL-12, etc...) and chemokines (IL-8, MCP, etc...). It is also implicated in LPS, CD40 or TLR-induced DC maturation. NFAT has been widely studied in T-cell activation where it plays a key role in antigen receptor-mediated responses by controlling the expression of a number of genes including cytokines such as IL-2, IL-4, TNF α , IFN γ and ligands such as CD40 and Fas ligand (reviewed by [40]). AP-1 proteins are regulated by PKC and Ras/Rac dependent pathways. AP-1 and NFAT transcription factors can form stable complexes that bind composite NFAT:AP-1 DNA sites. These composite sites have been identified in genes involved in the immune response such as IL-2, GM-CSF and IFN γ [41]. Activation of these transcription factors may reflect the diversity of bioactive

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molecules generated under PLA₂ treatment and may be an efficient way to integrate complex signals, enabling a fine tuning of biological responses. PLA₂-induced generation of mature DC probably results from the combined effects of several lipid mediators produced directly or indirectly by this enzyme. Among the various molecules resulting from PLA₂ activity, LPC was the best candidate to mediate PLA₂ effects on DC. Indeed, using the experimental procedures described above, we have shown previously that LPC is the major lipid mediator of oxidized LDL that can promote mature DC generation from differentiating monocytes by a G-protein coupled membrane receptor dependent pathway [22]. However, the transcription factors activated by LPC are different from those activated after bee venom PLA₂ treatment [30]. Therefore, LPC is unlikely to be directly responsible for the effects of PLA₂ on DC generation. Activation of NF-κB may be initiated by derivatives of both LPC and arachidonic acid. Further work is required to identify the lipid mediators and their signaling pathway.

Although bee venom sPLA₂ is a major allergen in humans, PLA₂-generated DC do not display a Th2-type function *in vitro* when cells are obtained from non-allergic donors. Accordingly, it is known for other allergens such as Der p 1, one of the major allergens of the house dust mite, that Th2 polarization of monocyte-derived DC is due to the allergic status of the donor [42]. It would therefore be interesting to compare the effects of PLA₂ on monocytes and DC from allergic versus non allergic individuals and to know whether environmental factors including histamine could reorient the function of PLA₂-generated DC toward a Th2-type as previously observed for LPS [43]. The overall data presented here suggest that sPLA₂ like bee venom or hGIII PLA₂ can generate lipid mediators with immunoregulatory functions *in vitro*. Increased PLA₂ activity during the APR may thus favor the development of the adaptive arm of immunity.

4 Material and methods

4.1 Differentiation of monocyte-derived dendritic cells

PBMC were isolated from human peripheral blood by density gradient centrifugation on Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden). Mononuclear cells were separated from PBL by centrifugation on a 50 % Percoll solution. Monocytes were purified by immunomagnetic depletion (Dyna, Oslo, Norway) using a cocktail of monoclonal Abs anti-CD19 (4G7 hybridoma), anti-CD3 (OKT3, ATCC, Rockville, MD, USA) and anti-CD56 (NKH1, Beckman Coulter, Fullerton, CA, USA). Monocytes were more than 90 % pure as assessed by CD14 labeling. Monocytes were differentiated to immature DC during 6 days with 40 ng/ml human recombinant GM-CSF and 250 U/ml human recombinant IL-4 in RPMI 1640 (Life Technologies, Rockville, MD, USA) supplemented with 2 mM glutamine (Life Technologies), 10 mM HEPES (Life Technologies), 40 ng/ml gentamycin (Life Technologies) and 10 % lipoprotein-deficient fetal calf serum (LPDS, Sigma, St Quentin-Fallavier, France). At the end of the differentiation (day 6), cells were harvested and analyzed. Cells were CD14⁺ CD1a⁺ and viability was superior to 90 %.

4.2 PLA₂ treatment

Bee venom PLA₂ (Sigma; min. purity 87%; 1360 U/mg protein; one unit will hydrolyze 1.0 μmole of soybean L-α-phosphatidylcholine per min at pH 8.9 and 25°C) was dissolved in 100 mM HEPES buffer pH 8.5, aliquoted and stored at -20°C. The catalytic domain of the human group III sPLA₂ was produced in insect cells and purified as previously described [28]. A 50X solution of PLA₂ was prepared extemporaneously by dilution in 100 mM HEPES / 5 mM CaCl₂ pH 8.5 buffer and 20 μl was added to 1 ml of differentiating DC at day 5. Cells were

incubated for 24 h with the indicated doses of PLA₂ or LPS (1 µg/ml). Control cells were incubated with 20 µl of buffer alone. At day 6, cells and supernatants were harvested and analyzed. When indicated, polymixin B (10 µg/ml; Sigma) was added to the culture 10 min before sPLA₂ (5 U/ml) or LPS (10 ng/ml) addition.

4.3 PLA₂ inactivation

sPLA₂ (250 U/ml) in HEPES-CaCl₂ buffer was heated at 95°C for 5 to 30 min and the enzyme was kept on ice until the assay. Enzyme activity was assayed by arachidonoyl-thio-PC hydrolysis (Cayman Chemicals, Ann Arbor, MI, USA) as described [44]. 5 µl of buffer (150 mM NaCl / 10 mM CaCl₂ / 80 mM HEPES / 4 mM Triton X-100 / 30 % glycerol / 1 mg/ml BSA pH 7.4) was added to 10 µl of heated PLA₂ and incubated for 60 min at room temperature with 200 µl of 1.5 mM arachidonoyl-thio-PC in the same buffer. After addition of 10 µl of DTNB/EGTA solution (25 mM DTNB / 475 mM EGTA / 0.5 M Tris-HCl pH 8) and incubation 5 min, the absorbance at 414 nm was measured against the blank without PLA₂. Enzymatic activity was measured in µmol of arachidonoyl-thio-PC hydrolyzed/min/ml.

4.4 Phenotype analysis

Phenotype was analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using FITC-conjugated anti-CD14, -HLA-DR, -CD80 and PE-conjugated anti-CD1a, -CD83, -CD86 all from Beckman Coulter.

4.5 Mixed leukocyte reaction (MLR)

PBMC were isolated by density gradient centrifugation on Ficoll-Hypaque. After depletion of monocytes on Percoll gradient, PBL were recovered in the dense fraction. T lymphocytes were purified by immunomagnetic depletion using a cocktail of monoclonal Abs anti-CD19 (4G7), anti-CD16 (3G8), anti-CD56 (NKH1), anti-glycophorin A (11E4B7.6) and anti-CD14 (RMO52) all from Beckman Coulter. T lymphocytes were more than 95 % pure as assessed by CD3 labeling. Primary MLRs were conducted in 96-well flat-bottom culture plates. DC were treated or not at day 5 with PLA₂, collected at day 6, extensively washed and resuspended in RPMI 1640 supplemented with 2 mM glutamine, 10 mM hepes, 40 ng/ml gentamycin and 10 % FCS (BioMedia, Boussens, France). Cells were then co-cultured with 2 x 10⁵ allogeneic T cells in 200 µl complete culture medium at 1/5, 1/10, 1/20 or 1/40 DC/T cells ratio. After 4 days, culture supernatants were tested for the presence of IFN γ by ELISA. To detect intracellular IL-4 and IFN γ , 2.10⁴ DC were co-cultured with 2.10⁵ allogeneic T cells for 5 days. Cells were restimulated with 100 U/ml recombinant IL-2 (Chiron) for 7 days. Cells were washed and incubated with 10 ng/ml PMA and 1 µg/ml ionomycin for 5h. 10 µg/ml brefeldin A was added for the last 2h. Cells were washed, fixed and permeabilized with cytofix/cytoperm solution (Becton Dickinson) and labeled with anti-IFN γ -FITC and anti-IL-4-PE (Becton Dickinson).

4.6 Cytokine measurement

Culture supernatants were aliquoted and stored at -80°C. IL-12p40 and IFN γ levels were determined using ELISA kits from Biosource (Camarillo, CA, USA) and Endogen (Woburn, MA, USA) respectively. IL-12p70 was assayed using the human Th1/Th2 Cytometric Bead Array system (BD Biosciences).

4.7 PLA₂ binding and internalization

Bee venom sPLA₂ (2 mg/ml) was labeled with Alexa488-protein labeling kit (Molecular Probes, Leiden, The Netherlands). Free Alexa488 was removed by gel filtration and labeled protein was recovered in PBS at 1.18 mg/ml.

The binding of Alexa488-PLA₂ was performed on differentiating cells harvested at day 5 and resuspended in culture medium. Alexa488-PLA₂ was incubated with cells (10^6 /ml) for 20 min on ice. In competition experiments, non labeled PLA₂ was added into the medium 10 min prior to incubation with Alexa488-PLA₂ on ice.

Internalization was carried out at 37°C for 2 to 20 min in RPMI / 10 % LPDS medium. To follow receptor-mediated endocytosis, cells were first incubated on ice for 20 min with 10 µg/ml Alexa488-PLA₂, washed twice at 4°C, and then incubated at 37°C for 10 min. Internalization was stopped on ice with cold PBS containing 0.1 % BSA and 0.05 % NaN₃. Cells were washed three times at 4°C in this buffer and analyzed by flow cytometry and fluorescence microscopy after cytospin and fixation. Slides were observed with the Leica DM IRB/E microscope or the Axioplan2 LSM510 Confocal microscope (Zeiss).

4.8 Electrophoretic Mobility Shift Assay (EMSA)

Differentiating cells were treated with 5 U/ml PLA₂ at day 5 for the indicated times (0, 2, 4, 8 or 24 h) as described above. After treatment, cells (4×10^6) were harvested and nuclear proteins were extracted: cells were washed twice with PBS, resuspended in 400 µl of ice-cold hypotonic buffer (10 mM Hepes (pH 7.9) / 10 mM KCl / 0.01M DTT / 1.5mM MgCl₂ / 1X protease inhibitor cocktail (Sigma)), left on ice for 10 min, vortexed and centrifuged at 15,000 g for 1 min at 4°C. Sedimented nuclei were resuspended in 40 µl ice-cold saline buffer (20

mM Hepes (pH 7.9) / 420 mM NaCl / 0.2 mM EDTA / 1.5 mM MgCl₂ / 25% glycerol / 1X protease inhibitor mixture (Sigma)), left on ice for 20 min, vortexed and centrifuged at 15,000 g for 5 min at 4°C. Nuclear protein concentrations were determined by Micro-BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The binding activity of nuclear proteins was determined using specific DNA probes as described [30]. Sequences of the double-stranded oligonucleotide used for detection of NF-κB: 5'-AGTTGAGGGGACTTTCCCAGG-3'; PPAR: 5'-GGGGTCAGTAAGTCAGAGGCCAGGGA-3'; AP-1: 5'-GTGACTCATGACTCATGACTCATGACTC-3' ; NFAT consensus: 5'-CGCCCAAAGAGGAAAATTTGTTTCATA-3'. Oligonucleotides were end-labeled with [γ -³²P]ATP (Amersham Biosciences) by T4 polynucleotide kinase (New England Biolab, Beverly, MA). For the binding reaction, 1 μg of nuclear extract was added to a reaction mixture containing 2 μg of poly (dI-dC) (Amersham Biosciences), 4 μl of 5X binding buffer (final concentration: 10 mM Tris (pH 7.5) / 50 mM NaCl / 1 mM DTT / 1 mM EDTA / 5% glycerol), and 50000 cpm of [³²P]-labeled oligonucleotide in a final volume of 20 μl and were incubated at room temperature for 20 min. Unlabeled competitor oligonucleotide was added in 50-fold excess to confirm the specificity of the binding reaction. The same amount of total protein was loaded on a 4% polyacrylamide non denaturing gel and DNA-protein complexes were separated from unbound DNA probe by electrophoresis in 0.5X Tris-glycine EDTA buffer. Gels were visualized using a Typhoon PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Image Master software (Amersham Biosciences) was used for quantification of band intensities that were normalized to non specific background.

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Figure legends

Fig. 1. Phenotypic maturation induced by bee venom PLA₂

Monocytes were differentiated in LPDS medium containing GM-CSF and IL-4 [22, 23]. Cells were treated or not at day 5 with PLA₂ or LPS and analyzed at day 6. A, Phenotype of cells treated with 5 U/ml PLA₂ (filled profile), 5 U/ml PLA₂ + polymixin (bold line), 10 ng/ml LPS (dotted line) or untreated control cells (thin line). B, Increasing concentrations of PLA₂ (0, 2, 5 or 10 U/ml) were added at day 5 and CD86 expression was analyzed at day 6. Data represent mean fluorescent intensities (mfi) normalized at 100 for control non-treated cells. Mean \pm S.D. of three experiments.

Fig. 2. Functional maturation induced by bee venom PLA₂

Monocytes were differentiated in LPDS medium containing GM-CSF and IL-4. Cells were treated or not at day 5 by addition of 5 U/ml PLA₂ or 1 μ g/ml LPS and harvested at day 6. A, Control immature DC or PLA₂-generated DC were harvested at day 6, washed and cultured for 4 days with allogeneic purified T cells (2×10^5 /well) at DC / T cell ratios ranging between 1/5 and 1/40. The amount of IFN γ in the supernatants of the coculture was measured by ELISA. B, Control immature DC, PLA₂-generated DC or LPS-treated DC were harvested at day 6, washed and cultured for 5 days with allogeneic purified T cells (2×10^5 /well) at 1/10 DC/T cell ratio. Cells were expanded with 100 U/ml IL-2 for 7 days, washed and restimulated with PMA and ionomycin for 5 hours. Brefeldin A was added for the last 2 hours and cells were processed for intracellular labeling with anti-IFN γ -FITC and anti-IL-4-PE.

Fig. 3. Phenotypic and functional maturation induced by the catalytic domain of hGIII-sPLA₂

Monocytes were differentiated in LPDS medium containing GM-CSF and IL-4. Cells were treated or not at day 5 by addition of 700 nM hGIII sPLA₂ and analyzed at day 6. A, Phenotype of cells treated or not with hGIII sPLA₂. Data represent mean fluorescent intensities normalized at 100 % for control non-treated cells. B, 10⁴ control immature DC or hGIII sPLA₂ treated DC were harvested at day 6, washed and cultured for 4 days with allogeneic purified T cells (2 x 10⁵/well). The amount of IFN γ in the supernatants of the coculture was measured by ELISA.

Fig. 4. Binding and internalization of PLA₂

Monocytes were differentiated in LPDS medium containing GM-CSF and IL-4 for 5 days. Cells were washed and resuspended in cold LPDS medium. A, Cells were incubated on ice for 20 min with increasing doses of Alexa488- PLA₂ (0, 4, 8, 16 or 32 μ g/ml). After washings, the amount of bound fluorescent PLA₂ was measured by flow cytometry. Data represent mean \pm S.D. from mean fluorescent intensities (mfi) of three experiments. B, Cells were incubated on ice for 20 min with increasing amounts of non labeled PLA₂ (20, 50, 100 or 200 μ g/ml) and 20 μ g/ml Alexa488-PLA₂. After washing, mean fluorescent intensity of cells was measured by flow cytometry. Data are expressed in percentage of PLA₂ binding without competitor. C, Cells were incubated with 2 μ g/ml Alexa488-PLA₂ at 37°C or on ice, washed and analyzed by flow cytometry. D, Cells were incubated with 4 μ g/ml Alexa488-PLA₂ on ice (up) or at 37°C (bottom), washed and observed in transmission (left) or fluorescence (right) microscopy. E, Receptor-mediated endocytosis: cells were incubated with 10 μ g/ml Alexa488-PLA₂ on ice for 20 min, washed and incubated at 37°C for 10 min. Cells were washed and analyzed by confocal microscopy.

Fig. 5. Phospholipase activity is essential to induce DC maturation.

PLA₂ in Hepes-CaCl₂ buffer was heated or not at 95°C for 5 to 30 min. A, Enzyme activity was assayed by arachidonoyl-thio-PC hydrolysis. B, Monocytes differentiating to DC were treated at day 5 with 4 µg/ml of PLA₂ heated for the indicated time. Cells were harvested at day 6 and their phenotype was analyzed. CD86 expression by PLA₂ treated cells was plotted against the heating time of PLA₂. C, Differentiating cells were washed at day 5, resuspended in cold LPDS medium and incubated for 20 min on ice with 4 µg/ml native PLA₂ or 4 µg/ml denatured PLA₂ (95°C for 30 min). Binding was measured as in figure 3.

Fig. 6. EMSA on nuclear extracts from PLA₂-generated DC.

Monocytes differentiating to DC were treated at day 5 with 5 U/ml PLA₂ for the indicated period of time. Cells were harvested and nuclear extracts prepared as described. Equal quantities of nuclear proteins were run for EMSA analysis, using ³²P-labeled DNA probes specific for NF-κB (A), AP-1, NFAT and PPARγ (B). The specificity of binding was shown by competition with non labeled probe in excess (lane C). C, Binding activity of the above transcription factors was determined by quantification of band intensities on gel shift assays using Image Master software.

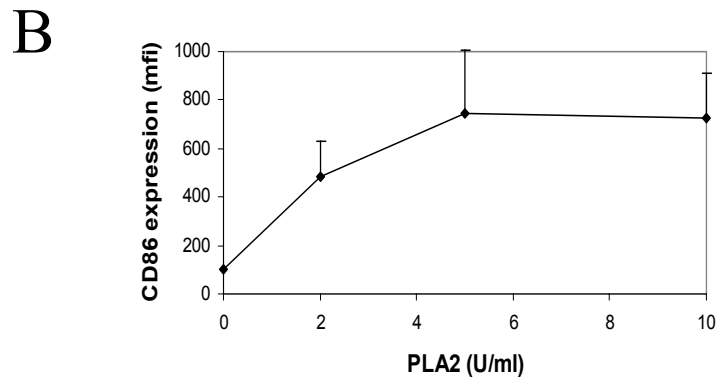
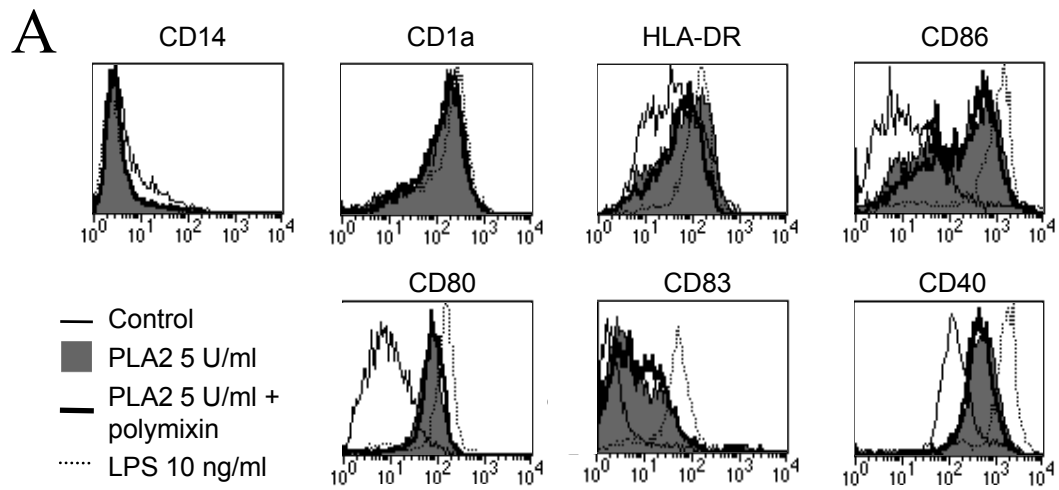


Figure 1

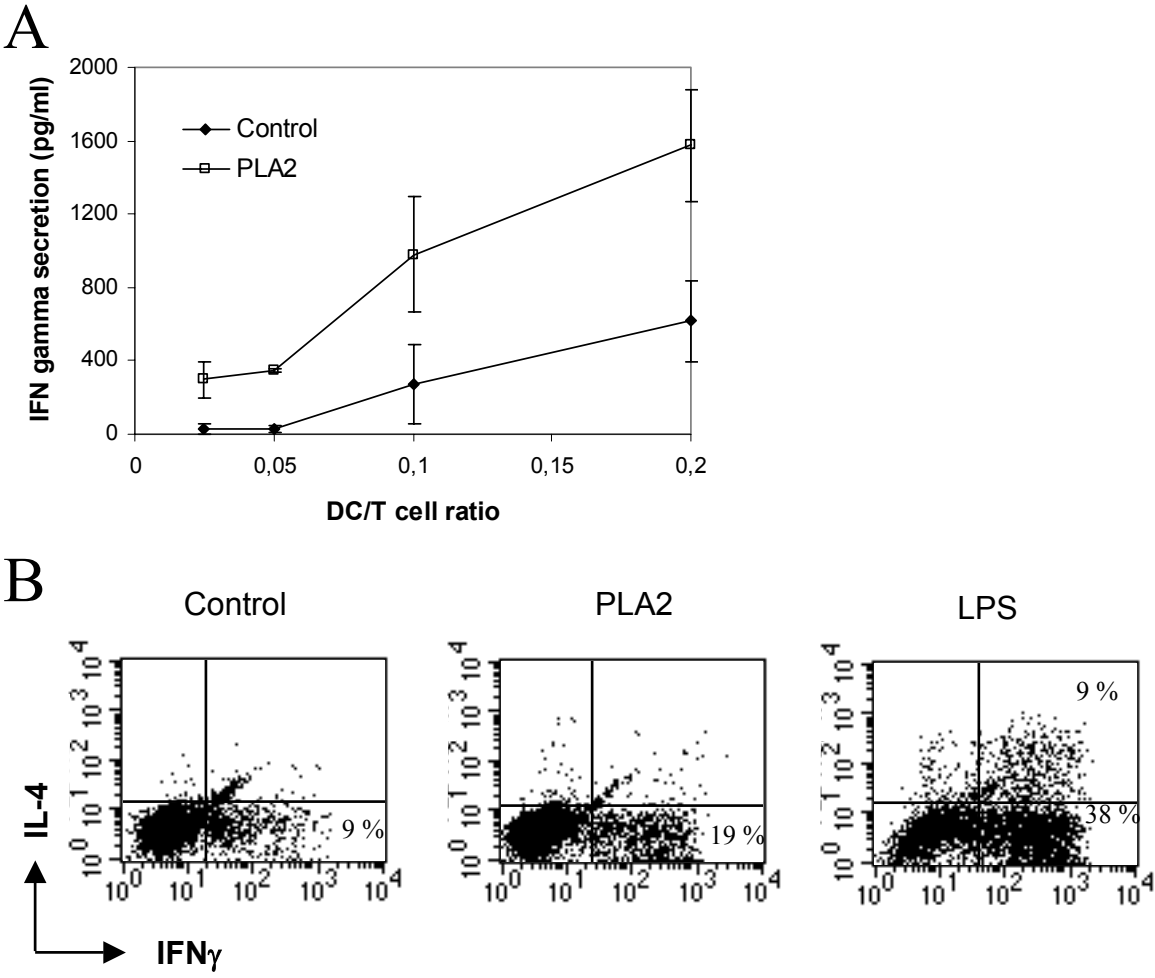


Figure 2

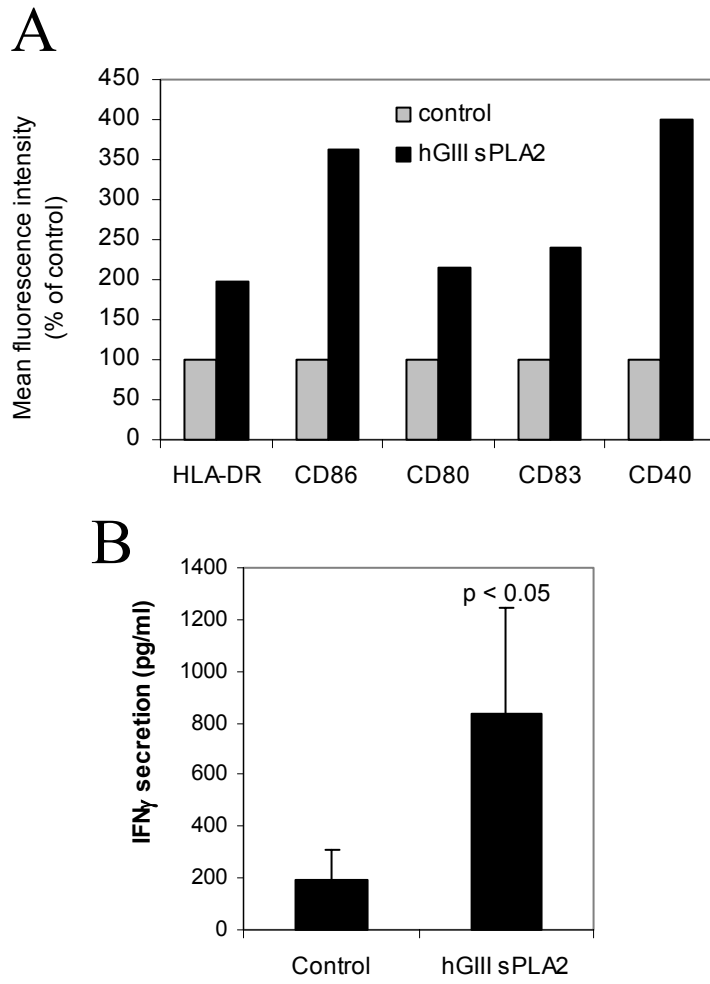


Figure 3

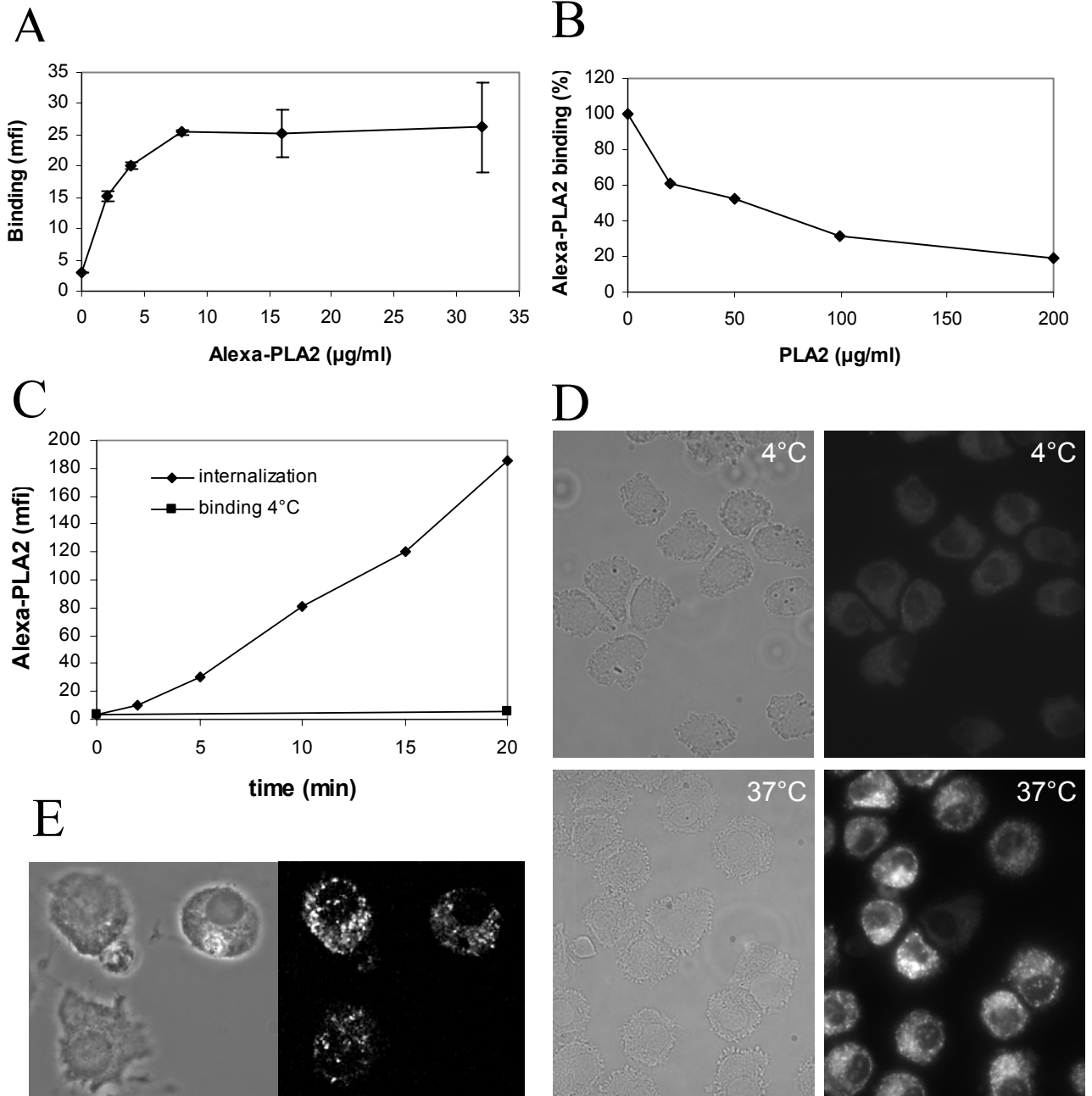


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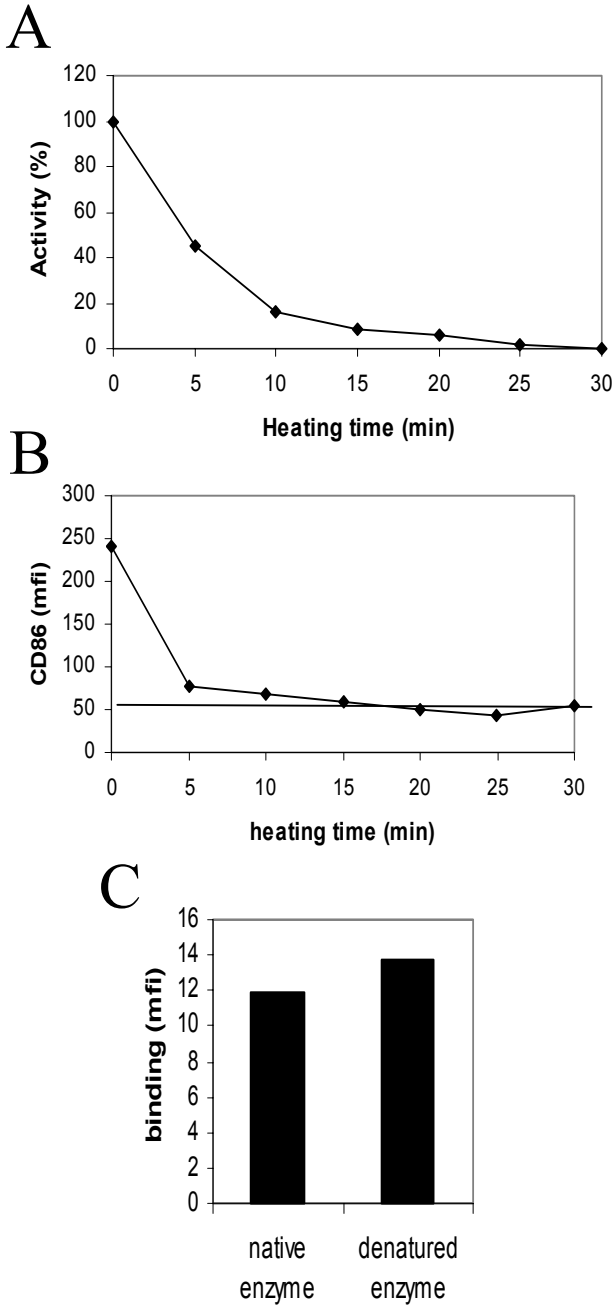


Figure 5

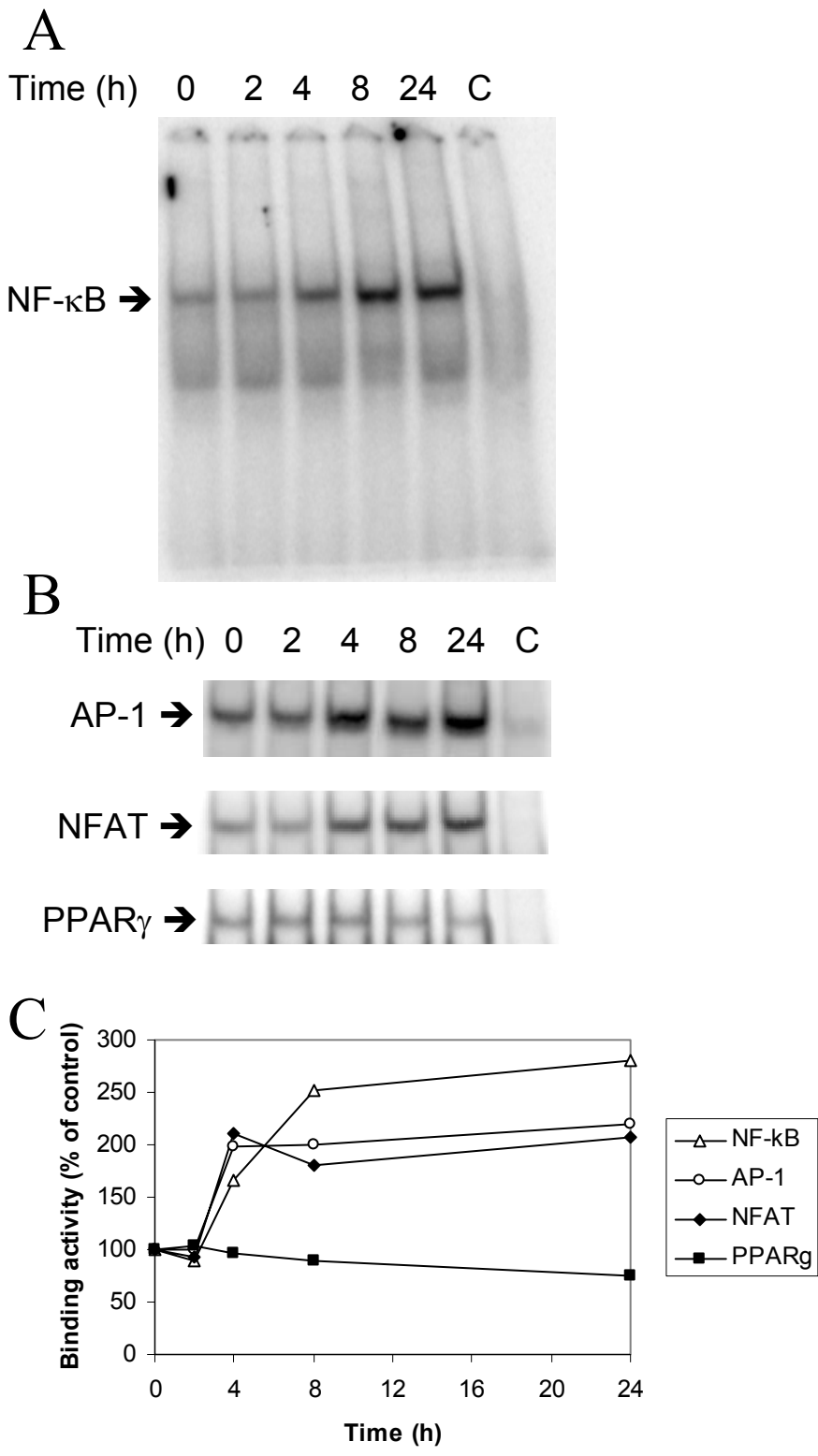


Figure 6