Cooperation Between Secretory Phospholipase A_2 and TNF-Receptor Superfamily Signaling

Implications for the Inflammatory Response in Atherogenesis

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Abstract—Atherogenesis is the consequence of a variety of effector mechanisms rather than the result of a single functional molecule. In this connection, type IIA secretory phospholipase A_2 ($sPLA_2$) is an acute-phase reactant, which accumulates in atherosclerotic arterial walls, elicits several effects on monocytes, and has been related to the development of atherosclerosis. CD40/CD40 ligand pair is also a strong proatherogenic system. $sPLA_2$ produced an increase of the surface expression of CD40 in THP-1 monocytes and enhanced the effect of CD40 ligation on the expression of both Fas and FasL, thus indicating the existence of a positive cooperation between $sPLA_2$ and different elements of the TNF-receptor superfamily. Activation of the CD40/CD40L dyad with anti-CD40 antibody produced a small release of arachidonic acid and lacked any significant effect on the induction of cyclooxygenase-2, whereas the secretion of the chemokine MCP-1 and the surface display of CD11b, the α chain of the integrin Mac-1, were upregulated. Engagement of CD40 did not influence the survival of THP-1 monocytes, but coincubation of THP-1 monocytes pretreated with anti-CD40 antibody and Jurkat cells induced a significant increase of the number of Jurkat cells showing binding of annexin-V, and nuclear condensation and fragmentation, thus indicating that this treatment might trigger a juxtacrine/paracrine mechanism of apoptotic death in sensitive cell types. This data indicates the existence of overlapping routes for the response to CD40, TNF-α, and $sPLA_2$, thus allowing the development of distinct patterns of response in monocytic cells. **(*Circ Res*. 2002;91:681-688.)**

Key Words: apoptosis ■ atherosclerosis ■ cytokines ■ inflammation ■ lipid mediators

The development of new concepts about atherogenesis in the context of inflammation has opened new vistas for the comprehension of this disease.[1] Recent reports have pointed to the dyad CD40/CD40 Ligand (CD40L) as a stimulus for atheroma-associated cells operating at all stages of atheroma development. CD40 is a cell-membrane spanning protein of about 50 kDa belonging to the tumor necrosis factor (TNF)-α receptor family, which also includes TNF receptors and Fas (CD95).[2,3] CD40 is constitutively expressed on B cells and participates in the regulation of cell proliferation, differentiation, and apoptosis.[4,5] The activator of CD40, CD40L, is a TNF-like molecule, the expression of which was originally considered as restricted to the surface of activated $CD4^+$ T cells, thus being associated to T cell–dependent B-cell responses and isotype switching. The CD40/CD40L system is now recognized as widely distributed, and it has recently been reported that vascular cells and macrophages express functional CD40L, as well as its receptor CD40 in atherosclerotic plaques.[6] Engagement of CD40 endows cells with functions that mediate inflammatory responses. Consequently, CD40 signaling has been associated not only with atherosclerosis, but also with the pathogenesis of other inflammatory diseases. CD40 has been related to the modulation of various stages of atherogenesis[7]: initiation, evolution, and rupture of the plaque. In the early phase, CD40 participates through the induction of adhesion molecules and the release of chemokines. In established lesions, its function includes the production of proinflammatory cytokines. In regard to the stability of atherosclerotic plaque, CD40 ligation induces the synthesis of matrix-degrading enzymes and prothrombotic activities.[8,9]

Another molecule associated with the development of atherosclerotic lesions is the secreted type IIA phospholipase A_2 ($sPLA_2$), because it has been detected in all stages of human atherosclerotic arteries,[10,11] and transgenic mice hyperexpressing this enzyme develop severe atherosclerosis and display lipoprotein profiles more proatherogenic than those of nontransgenic littermates.[12] Immunohistochemical studies indicate $sPLA_2$ is present in smooth muscle cells (SMCs) from both the media and intima layers and in macrophage-rich

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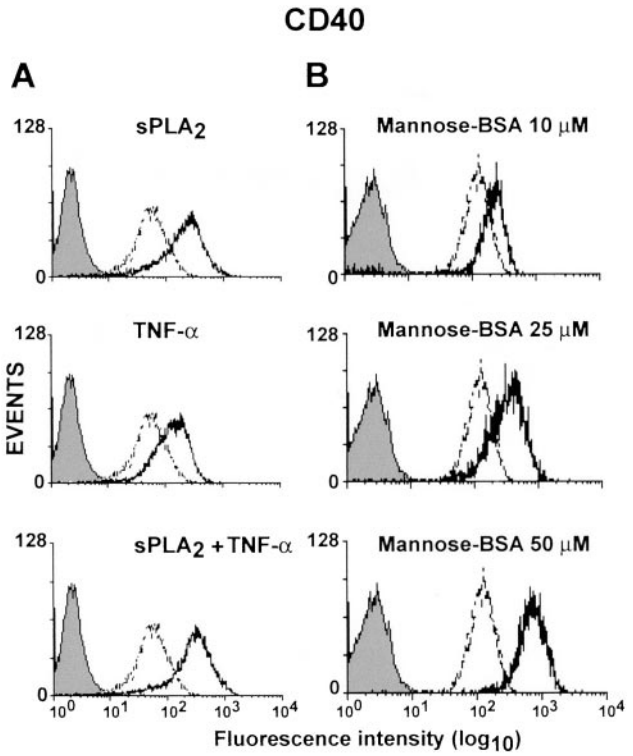

Figure 1. Effect of different additions on the expression of CD40 in THP-1 cells. Cells were incubated for 18 hours with TNF-α (100 U/mL), $sPLA_2$ (1 μg/mL), and mannose-BSA as indicated. Each panel is a histogram representing cell number (*y* axis) vs fluorescence intensity (*x* axis) for 10 000 cells. CD40 was detected with mAb 5C3 IgG_1 and in each panel is compared with the binding of irrelevant antibody (shown in solid gray). Open black histograms (wide tracing) represent stimulated cells and open histograms (narrow tracing) represent resting cells. Results shown are representative of 3 independent experiments.

regions of atherosclerotic plaques, making SMCs the main source of the enzyme. In addition, it has been reported that proinflammatory cytokines present in atherosclerotic lesions can regulate $sPLA_2$ transcription and secretion by arterial wall.[13] Regarding the monocyte-derived macrophage contribution, in vitro studies have shown the induction of $sPLA_2$ by mildly oxidized low-density lipoprotein (LDL).[14] To date, some of the biological activities of $sPLA_2$ have been attributed to its enzymatic capacity to hydrolyze phospholipids. However, several lines of evidence suggest that some of the physiological actions of $sPLA_2$ are not due to its hydrolytic activity, but to specific binding to cell surface receptors. In fact, reverse transcription-polymerase chain reaction (RT-PCR) analyses have shown the presence of mRNA for the M-type $sPLA_2$ receptor in bone marrow–derived mast cells, THP-1 cells, and human monocytes,[15–17] and it has been shown that $sPLA_2$ activates biochemical pathways such as the MAP-kinase cascade and the cytosolic phospholipase A_2 ($cPLA_2$), thus leading to arachidonate release and to a variety of biological effects including cell migration, mitogenesis, and cytokine release.[16–20] This study shows that activation of monocytic cells by $sPLA_2$ elicits the surface display of functional CD40, thus enlarging the scope of biological responses that these cells may undergo during atherogenesis.

Materials and Methods

Reagents and Cell Culture

$sPLA_2$ was purified from plasma of patients diagnosed of sepsis as previously described.[21] This procedure provides a unique protein band, the identity of which has been ascertained by Edman's N-terminal sequencing. Written informed consent was obtained from patients. The absence of lipopolysaccharide (LPS) in the preparation was confirmed by the *Limulus amebocyte* lysate assay test in the batches used for the experiments. Moreover, experiments are conducted in the absence of FBS, which ensures that the effect is observed in the absence of LPS binding protein, which is necessary for the action of low concentrations of LPS. Goat anti-cyclooxygenase-2 (COX-2, C-20) and mouse monoclonal anti-$cPLA_2$ (4-4B-3C) were from Santa Cruz Biotechnology (Santa Cruz, Calif). THP-1 and Jurkat cells were cultured in RPMI 1640 (GIBCO) supplemented with 2 mmol/L glutamine and 5% heat-inactivated FBS. Cells were rested for 24 hours before the experiment, and then were stimulated for the indicated times with 2 μg/mL anti-CD40 mAb, 100 U/mL TNF-α, 1 μg/mL $sPLA_2$, or combinations of agonists. The detection of monocyte chemoattractant protein-1 (MCP-1) was carried out by ELISA with reagents from R&D Systems, Inc, as described.[22]

Flow Cytometry

THP-1 cells were assayed for CD11b, CD40, CD40L, Fas, and FasL expression. For this purpose, 5×10^6 cells/dish were treated with the agonists for 18 hours. The cells were collected, suspended in PBS supplemented with 1% BSA and incubated with 10 μg/mL anti-human CD11b/MAC-1 IgG_1 mAb, 10 μg/mL anti-human CD40 5C3 IgG_1 mAb, 10 μg/mL anti-human CD40L TRAP1 IgG_1 mAb, 1 μg/mL anti-human FasL NOK-1 IgG_1 mAb, or 500 ng/mL anti-human Fas DX2 IgG_1 mAb (Pharmingen) for 1 hour at 4°C. After washing with PBS, goat anti-mouse IgG-FITC conjugate (Sigma) 1:100 was added and incubated for 30 minutes at 4°C. Subsequently, cells were washed and analyzed by immunofluorescence flow cytometry in a FACScan cytofluorometer (Becton Dickinson). Positive cells were estimated using P3×63 myeloma supernatant as a negative control or an isotype-matched control negative Ab at an equivalent concentration. To increase the levels of membrane-bound FasL by blocking FasL cleavage before the stimulation with the agonists, cells were treated with 10 μmol/L of the matrix metalloproteinase inhibitor K8301 (Pharmingen). Data were analyzed using CellQuest software (Becton Dickinson)

Cell Cycle Analysis

THP-1 cells were incubated for 24 hours with the agonists, washed twice with cold PBS, and fixed with 70% ethanol. After overnight incubation at 4°C, cells were washed and resuspended in PBS/5 mmol/L EDTA. RNA was removed by digestion with RNase A at room temperature. After 1 hour of incubation with 0.5 mL of staining solution (500 μg/mL propidium iodide in PBS/5 mmol/L EDTA), cell cycle analysis was performed by flow cytometry.

Cell Proliferation and Apoptosis Assays

THP-1 cells were starved overnight, and after 24 hours of stimulation, cells were pulsed with 1 μCi [^{3}H]thymidine for 4 hours before harvesting and counting of radioactivity. For apoptosis assay, cells were starved overnight, and then washed and incubated with the different agonists. After 48 hours stimulation, apoptosis was measured with the annexin-V FITC apoptosis detection kit of Pharmingen. Apoptosis was also evaluated by DAPI staining. For this purpose, cells were fixed with 4% paraformaldehyde and then permeabilized by 0.5% Triton-X-100 in PBS. After permeabilization, cells were stained for 30 minutes with DAPI (1 μg/mL) and analyzed by fluorescence microscopy to assess chromatin condensation.

Cell Fatty Acid Release

Subconfluent cells were starved overnight and labeled for 3 hours with 0.30 μCi/mL [^{3}H]arachidonic acid ([^{3}H]AA) or with 0.30

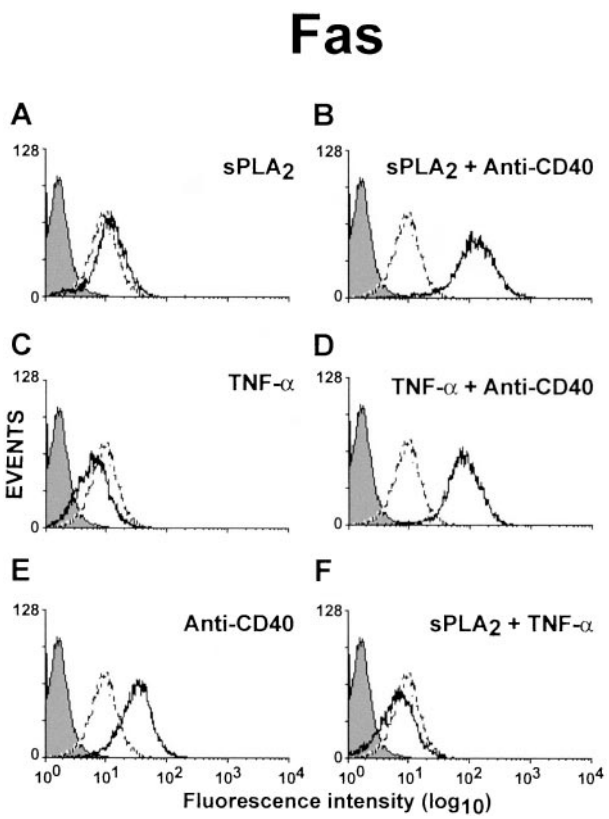

Figure 2. FACS analysis of THP-1 cells stained for Fas (open curves) or for unspecific isotype-matched control Ab (solid gray curves). Cells were stimulated 18 hours in the presence of TNF-α (100 U/mL), sPLA$_2$ (1 μg/mL), anti-human CD40 mAb (2 μg/mL), or combination of agonists. Open black histograms (wide tracing) represent stimulated cells and open histograms (narrow tracing) represent resting cells. Mean fluorescence intensity for control cells was 11±2 AU vs 39±4 in anti-human CD40 treated cells, 161±14 in cells treated with both anti-human CD40 and sPLA$_2$, and 95±7 in cells treated with TNF-α and anti-human CD40. This is a representative experiment of 3 similar ones.

μCi/mL [^{14}C]oleic acid ([^{14}C]OA). Cells were then washed with RPMI containing 0.2% BSA and stimulated for 30 minutes with different stimuli. Medium was removed and released radioactivity in supernatants was quantified by liquid-scintillation counting. The cells were solubilized with 1% Triton X-100 to measure the total amount of [^{3}H]AA or [^{14}C]OA incorporated into the cellular phospholipids.

Immunodetection of cPLA$_2$ and COX-2 Proteins

THP-1 cells were lysed in an ice-cold lysis buffer, and 50 μg of cellular protein from each sample were separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Dilution of both primary and secondary Ab was made in PBS containing 5% defatted dry milk and 0.1% Tween 20. After 1 hour of incubation with the respective primary antibody 1:1000, blots were washed 4 times and the secondary peroxidase-conjugated antibody was added for another hour. Immunoblots were developed using the Amersham ECL system.

Reverse Transcription-Polymerase Chain Reaction

Total cellular mRNA was extracted by the TRIzol method (Life Technologies) according to the manufacturer's instructions. cDNA was prepared by reverse transcription of RNA and then was amplified by PCR according to the following conditions: 1 cycle of initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, primer annealing for 30 seconds at 55°C, and extension at 72°C for 30 seconds, then 1 cycle of final extension at 72°C for 7 minutes. The PCR primers for human MCP-1 were 5′-GGATCCATGAAAGTCTCTGCCGCCCT-3′ (sense) and 5′-GAATTCTCAAGTCTTCGGAGTTTG-3′ (antisense). The expected PCR product, analyzed by ethidium bromide staining in 1.5% agarose gels was 300 bp for MCP-1. The expression of β-actin was used as control for the assay of a constitutive expressed gene.

Results

Connections of the TNF Receptor/Ligand Superfamily, CD40/CD40L, and Fas/FasL to the sPLA$_2$ Signaling Pathway in THP-1 Monocytes

Because both the CD40/CD40L dyad and sPLA$_2$ have been detected in human atheroma lesions, we addressed the possible link between both systems by looking at the effect of sPLA$_2$ on CD/CD40L in the monocytic cell line THP-1. These cells constitutively express CD40 at their surface, but not CD40L. Stimulation with both TNF-α and sPLA$_2$, at concentrations similar to those detected in plasma after injection of bacteria,[23] led to an upregulation of CD40 expression (Figure 1A); however, these agonists showed differences in the intensity of the induction, with sPLA$_2$ being most effective, reaching a mean fluorescence intensity of 295±42 (mean±SD) versus 155±24 AU for TNF-α ($P<0.01$). Interestingly, combination of both agonists elicited a higher effect (355±19 AU; $P<0.05$). The effect of sPLA$_2$ was not affected by preincubation with polymyxin B, thus ruling out a role for contamination by LPS of the sPLA$_2$ batch. Because many effects of sPLA$_2$ are produced by binding to the M-type receptor, which belongs to the C-type multilectin mannose receptor family, we assessed the effect of *p*-amino-phenyl-α-D-mannopiranoside-BSA (mannose-BSA), a noncatalytic ligand of the M-type receptor. As shown in Figure 1B, overnight treatment with different doses of mannose-BSA led to CD40 upregulation to a similar extent to that induced by sPLA$_2$, thus suggesting that sPLA$_2$ receptor occupancy, rather than the catalytic activity of sPLA$_2$, triggers CD40 expression. In contrast, all of these treatments failed to influence the expression of CD40L (data not shown).

Because the Fas/FasL dyad is influenced by sPLA$_2$,[16] and some of its functions involve cooperation with CD40,[24,25] we next determined the effect of CD40 ligation on Fas/FasL induction by using anti-CD40 antibody as a cross-linking reagent for CD40. Overnight treatment of THP-1 cells with 2 μg/mL anti-CD40 resulted on the surface display of both Fas (Figure 2E) and FasL (Figure 3E). The expression of Fas was further increased when cells were coactivated with anti-CD40 together with sPLA$_2$ or TNF-α (Figures 2B and 2D), while these stimuli on their own did not influence the surface display of Fas (Figures 2A, 2C, and 2F). Furthermore, although TNF-α did not influence the resting levels of the dyad Fas/FasL, TNF-α significantly enhanced the effect of anti-CD40 (Figures 2D and 3D), thus pointing to a cooperative effect of these elements of the TNF receptor/ligand superfamily.

Effect of CD40 Engagement on [^{3}H]AA Mobilization

To address the functional significance of CD40 expression on arachidonate metabolism, we determined the effect of CD40

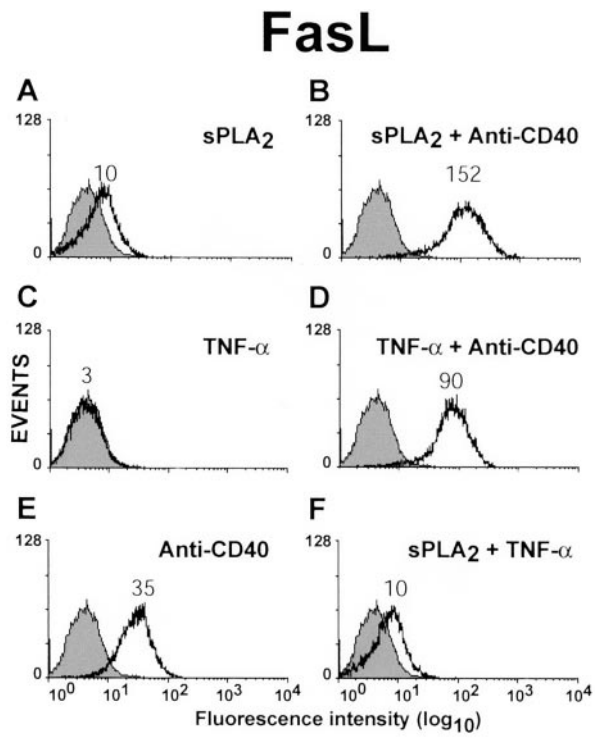

Figure 3. FACS analysis of THP-1 cells stained for FasL (open curves) or for unspecific isotype-matched control Ab (solid gray curves). Cells were stimulated 18 hours in the presence of TNF-α, $sPLA_2$, anti-human CD40, or combination of agonists. FasL expression was not observed in resting cells and its curve overlaps with the unspecific control Ab. Figures on top of the open curves indicate the mean fluorescence intensity values. Representative histograms of 3 independent experiments are shown.

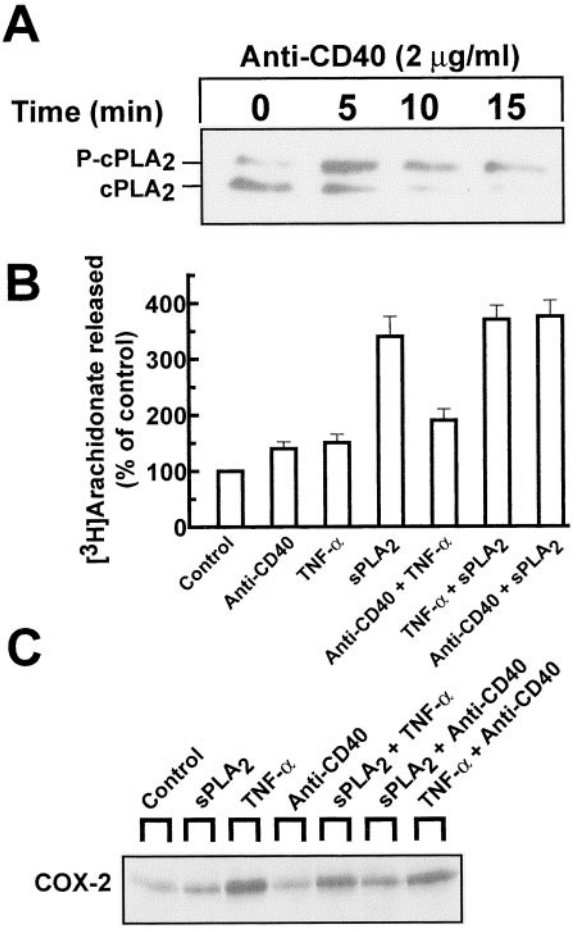

Figure 4. Effect of CD40 stimulation on the $cPLA_2$ system and arachidonate metabolism. THP-1 cells were incubated with 2 μg/mL anti-human CD40 mAb for the times indicated and then cell lysates were collected to assay the band-shift characteristic of $cPLA_2$ phosphorylation (A). Cells labeled with [^{3}H]AA were stimulated with TNF-α, $sPLA_2$, 50 μmol/L mannose-BSA, anti-human CD40 mAb, or combination of agonists for 30 minutes, and then [^{3}H]AA release was measured. Data represent mean$\pm$SD of 5 independent experiments (B). Cells treated under these conditions for 18 hours were collected for the immunodetection of COX-2. This is a representative experiment of 4 similar ones (C).

ligation on $cPLA_2$ activation and COX-2 induction. As shown in Figure 4A, stimulation of CD40 for 15 minutes induced an almost complete phosphorylation of $cPLA_2$, as judged from the appearance of a shift in the electrophoretic mobility, which is due to the phosphorylation of the enzyme by MAP kinase and is associated with its activation. We next assessed whether $cPLA_2$ activation was accompanied by [^{3}H]AA release. As shown in Figure 4B, $sPLA_2$ was the most potent agonist, and its effect was only slightly increased by both CD40 and TNF-α. To investigate whether the production of [^{3}H]AA in response to $sPLA_2$ is due either to its enzymatic activity or to binding to its membrane receptor, some experiments were performed in cells labeled with [^{14}C]OA, because $sPLA_2$ does not display specificity toward the fatty acid esterified at the *sn*-2 position of phospholipids. Under these conditions, no net release of [^{14}C]OA was observed (data not shown), which strongly suggests that $sPLA_2$ is acting through a signaling mechanism involving the recruitment of a PLA_2 specific for [^{3}H]AA and is consistent with its reported action through the activation of $cPLA_2$. Moreover, mannose-BSA, a ligand of $sPLA_2$ receptor, induced a [^{3}H]AA build-up similar to that induced by $sPLA_2$ (Figure 4B). The induction of COX-2 protein was assessed after stimulation with anti-CD40 for 18 hours. Unlike $sPLA_2$ and TNF-α, anti-CD40 did not upregulate COX-2 expression, and combination of agonists only resulted in a mild enhancement of the response (Figure 4C). Moreover, shortest times of incubation with anti-CD40 provided the same negative results (data not shown).

CD40 Ligation Enhances the Proapoptotic Effect of THP-1 Cells on Coculture With Jurkat Cells and Upregulates CD11b Expression

CD40 stimulation produces a variety of responses related to cell survival, which depend on the cell type. The effect of triggering CD40 on [^{3}H]thymidine incorporation was assessed to address any possible mitogenic effect. However, this treatment did not induce any significant change in [^{3}H]thymidine incorporation (30 632$\pm$2312 dpm/10^6 in control cells versus 32 214$\pm$3976 dpm/10^6 cells after anti-CD40 n=5), whereas FCS resulted in an incorporation of 55 659$\pm$6150 dpm/10^6 cells. As shown in Figure 5A, most resting THP-1 cells (80%) are found in the G_i/G_o phase of the cell cycle, and this was not significantly affected by incuba-

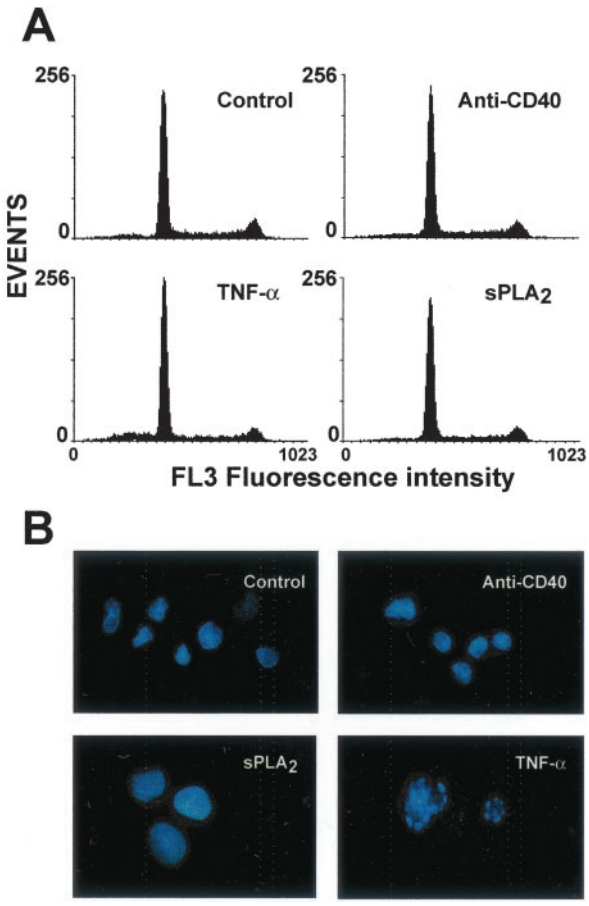

Figure 5. Effect of different additions on the proliferation/apoptosis of THP-1 cells. Cells were treated with TNF-α, sPLA$_2$, or anti-human CD40 mAb. After 24 hours, cells were fixed and permeabilized. DNA was stained with propidium iodide and analyzed by flow cytometry (A). In parallel experiments, cells were used for staining of nuclei with DAPI and assay of the nuclear morphology of apoptosis. Data from 1 of 3 representative experiments with similar results are shown (B).

tion with anti-CD40 for 24 hours. Experiments with DAPI staining showed nuclear morphological alterations typical of apoptosis in THP-1 cells stimulated with TNF-α, whereas these changes were not observed in cells treated with other agonists (Figure 5B). Because CD40 ligation does not influence the apoptotic death on THP-1 cells, even though it increases Fas and FasL expression (Figure 2E and 3E), it was addressed whether CD40-induces juxtacrine signaling on T cell survival. These studies were performed taking advantage of the expression of CD11b in THP-1 cells, which is absent in Jurkat cells, thus allowing the discrimination of the two cell populations in the coculture (Figure 6A). Interestingly, CD40 ligation affected CD11b expression on THP-1 cells (Figure 6A, top panels), because overnight treatment with anti-CD40 increased the surface display of CD11b protein, as judged from the shift to the right (white peak) in comparison with the resting situation (gray peak). This effect was also observed in the coculture, thus allowing a better distinction of the two cell populations (Figure 6A, bottom panel). To evaluate the occurrence of cell death induced on Jurkat cells by THP-1 cells pretreated with anti-CD40, the area under the marker that belongs to the Jurkat T cells was analyzed for annexin-V binding (Figure 6A, bottom panel). The annexin-V staining for Jurkat cells and THP-1 cells reveals few apoptotic cells with no increase after CD40 ligation (Figure 6B, top panels). The same assay was performed in the cocultures using resting THP-1 or anti-CD40–treated THP-1 cells as stimulus for Jurkat cells. Although resting THP-1 cells induced a small binding of annexin-V in Jurkat cells (24.50$\pm$4.37%), when THP-1 cells had been prestimulated 9 hours before the start of the coculture, the percentage of Jurkat cells expressing annexing-V increased to 49.09$\pm$8.12%, (mean$\pm$SD, n=6, $P<0.001$; Figure 6B, bottom panels). Figure 6C shows the cocultures stained with anti-CD11b (green) and DAPI (blue). In Figure 6Cb, THP-1 cells have been prestimulated and CD11b negative cells (Jurkat cells) showed the nuclear condensation and fragmentation typical of apoptosis. However, in the presence of resting THP-1 cells (Figure 6Ca) nuclear alterations were not observed.

CD40 Ligation Upregulates the Mechanism of Monocyte Recruitment

Monocyte recruitment to the inflammatory foci is mediated by strong interactions with the endothelium involving the β_2-integrin family of receptors, as well as by the concomitant activation by chemoattractants. As mentioned before (Figure 6A), CD40 engagement upregulates the surface display of the CD11b chain of the β_2-integrin Mac-1, whereas no effect was observed with both TNF-α and sPLA$_2$ (not shown). In keeping with an additional effect of CD40 on monocyte recruitment, THP-1 cells activated with anti-CD40 showed and enhanced expression of MCP-1 mRNA, which was accompanied by an increase of MCP-1 protein (Figures 7A and 7B). Unlike the effect on CD11b expression, this response was also enhanced by TNF-α and sPLA$_2$. Because κB-dependent transcription is one of the mechanisms involved in the regulation of MCP-1 expression, the possible effect of CD40 engagement on NF-κB activation was searched by looking at the degradation of the inhibitory proteins IκBα, IκBβ, and IκBϵ. However, treatment of THP-1 cells with anti-CD40 Ab did not produce any effect on these proteins (data not shown), which indicates that the CD40/CD40L dyad does not activate the NF-κB route in THP-1 monocytes.

Discussion

The role of the CD40/CD40L dyad in atherogenesis has been highlighted by a number of significant findings: (1) the colocalization of both CD40 and CD40L in atheroma-associated cells; (2) the induction of the expression of proatherogenic mediators in vitro by engaging this system[26–30]; and (3) the production of atherogenesis in vivo by acting on the system.[31–33] Moreover, the connection of the CD40/CD40L system with proinflammatory cytokines has been established by several findings. Thus, CD40 induction by cytokines has been reported in macrophages,[34] keratinocytes,[35] vascular endothelium,[36] and aortic smooth muscle cells, where this effect is elicited by the NF-κB route.[26] In this study, we have shown that the CD40/CD40L system is also linked to the sPLA$_2$ signaling pathway, which in view of the reported proatherogenic effects of sPLA$_2$,[37] is an argument in

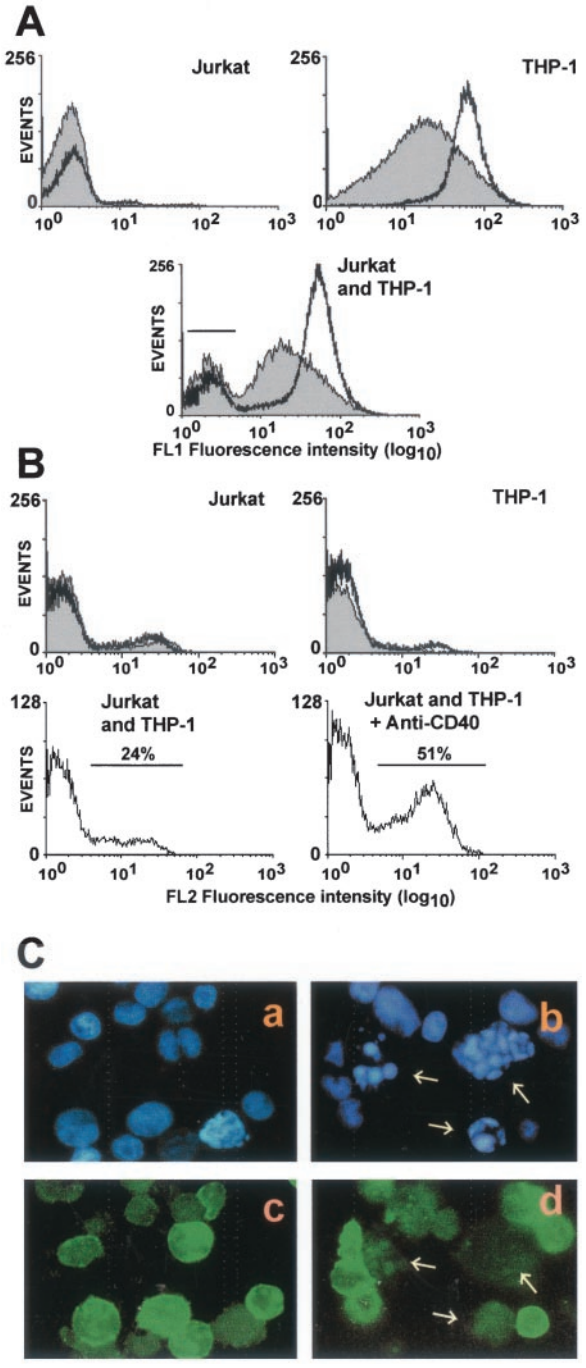

Figure 6. Effect of CD40-activated THP-1 cells on apoptosis of Jurkat cells. Jurkat and THP-1 cells were incubated for 18 hours in absence or in the presence of 2 μg/mL anti-CD40 mAb, and then used for immunostaining for CD11b (FL1 fluorescence) and annexin-V (FL2 fluorescence). In coculture panels, resting Jurkat cells were incubated with either resting or CD40-stimulated THP-1 cells. In A, solid histograms represent resting cells and open histograms represent stimulated cells analyzed by flow cytometry. Bar on the top in the coculture panel (A, Bottom) marks the Jurkat cell population with negative fluorescence for CD11b gated for the detection of annexin-V. In B, solid histograms represent resting cells and open histograms represent stimulated cells (Top). Bottom, Effect of the coculture on the expression of annexin-V on the gated cell population of Jurkat cells. On top of the bars is indicated the percentage of cells expressing annexin-V. C, To assay the nuclear morphology of apoptosis, cocultures with either resting (a and c) or stimulated (b and d) THP-1 cells were fixed and stained with anti-CD11b (c and d) and DAPI (a and b) and analyzed by fluorescence microscopy (final magnification $\times$40).

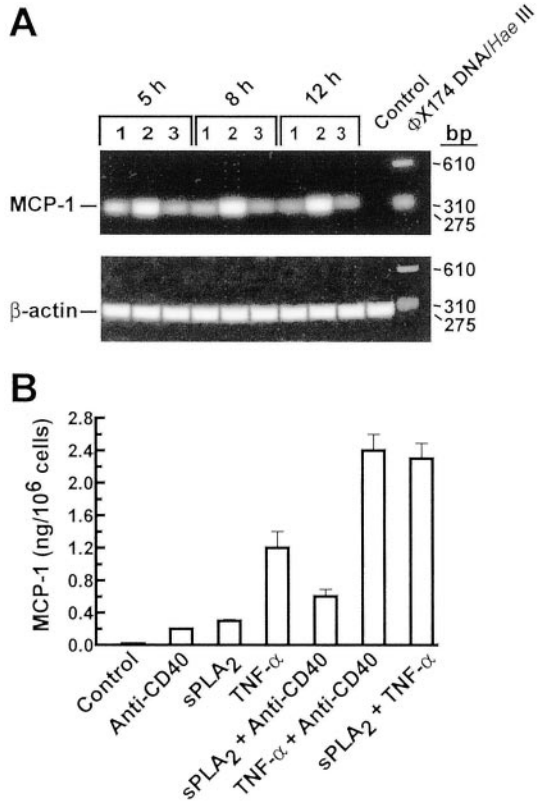

Figure 7. Effect of different agonists on the expression of MCP-1. Total mRNA was obtained from THP-1 cells incubated for different times with $sPLA_2$ (1), TNF-α (2), or anti-human CD40 mAb (3). RT-PCR reactions resulted in the production of MCP-1 cDNA product of the expected size (A). Cells were treated with TNF-α, $sPLA_2$, or anti-human CD40 mAb or combination of agonist for 24 hours and the production of MCP-1 protein was assayed in the supernatants (B).

favor of a cooperative interaction between both molecules in atherogenesis. However, the signaling pathways triggered by the CD40/CD40L system differ among distinct cell types, and little information exists regarding the functional consequences of the modulation of this system in macrophages as yet.

The present data show that ligation of CD40 on monocytes initiates a series of proinflammatory events that includes (1) activation of enzymes of arachidonate metabolism, (2) increased expression of molecules involved in the recruitment of mononuclear cells, and (3) upregulation of molecules that regulate cell death. In fact, signaling through CD40 in THP-1 cells activates $cPLA_2$, the enzyme that selectively hydrolyzes arachidonic acid from membrane phospholipids, and allows its conversion into compounds with functional relevance for atherogenesis, in view of the role that eicosanoids exert on atherogenesis through stimulation of intracellular signal transduction[38–41] and leukocyte recruitment.[42] In contrast, no detectable effect on the expression of COX-2 was observed, which differs from the results reported in lung,[43] orbital,[44] and human fibroblasts, where the CD40-CD40L bridge upregulates the expression of COX-2.

Consistent with a role for CD40 in the recruitment of leukocytes to the plaque is the regulation of the expression of adhesion molecules, as judged from the increased surface

display of CD11b. In fact, reduction of CD11b expression has been associated to the protective effect of a leukotriene B_4 antagonist in the progression of atherosclerotic lesions in mice.[42] Because it has been shown that ligation of CD40 on endothelial cells by activated T cells expressing CD40L upregulates ICAM, VCAM, and E-selectin[30] and mediates signals that induce the secretions of leukocyte chemoattractans,[45] the CD40/CD40L system influences leukocyte migration by at least two mechanisms: modulation of CD11b interaction with its cognate ligands and secretion of MCP-1.

The downstream consequences of CD40 ligation are strongly dependent on the cell type. Thus, sustained AP-1 activation signaling has been proposed as the mechanism involved in Fas-depending apoptosis after CD40 engagement in human intrahepatic biliary epithelial cells,[46] and CD40 activation induces apoptosis via induction of the cell surface FasL expression in human hepatocytes.[47] As shown in this study, stimulation of THP-1 cells via CD40 does not produce apoptosis, even though the expression of both Fas and FasL are increased. This irrelevant effect of CD40 signaling on monocyte survival is similar to that previously found for sPLA$_2$,[16] although it might contribute to the development of Fas/FasL-dependent apoptosis in sensitive cell types.

Taken together, this data indicate the existence of different routes for the response to CD40, TNF-α, and sPLA$_2$. Because they overlap many of the signaling pathways, their cooperative action may provide a distinct profile of responses that might favor the most adequate functional pattern. A corollary to the aforementioned findings is that sPLA$_2$ might exert a portion of its proinflammatory effects through the modulation of CD40 expression. Cooperative interaction between these molecules may provide an amplification loop with a dualistic effect initially directed to trigger the production of lipid mediators and leukocyte recruitment, and then followed by a mechanism to eliminate such cells.

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