Cell. Mol. Life Sci. 65 (2008) 620 – 630
1420-682X/08/040620-11
DOI 10.1007/s00018-007-7520-1

Cellular and Molecular Life Sciences

Research Article

DiC14-amidine confers new anti-inflammatory properties to phospholipids

C. Lonez, A. Legat, M. Vandenbranden and J.-M. Ruysschaert*

Center of Structural Biology and Bioinformatics, Laboratory of Structure and Function of Biological Membranes, Université Libre de Bruxelles, Campus Plaine CP 206/2, 1050 Brussels (Belgium), Fax: +32-2-650-5382, e-mail: jmruyss@ulb.ac.be

Received 12 November 2007; received after revision 4 December 2007; accepted 4 December 2007
Online First 22 December 2007

Abstract. The inflammatory effect of unmethylated CpG DNA sequences represents a major obstacle to the use of cationic lipids for *in vivo* gene therapy. Although the mechanism of CpG-induced inflammatory response is rather well understood nowadays, few solutions have been designed to circumvent this effect in gene therapy experiments. Our previous work has shown that a refractory state towards inflammation can be elicited by preinjecting cationic liposomes. Here, we present evidence that diC14-amidine liposomes confer new anti-inflammatory properties to phospholipids from low-density lipoprotein (LDL) and even to synthetic phospholipids for which such an observation has not been reported so far. Whereas oxidation of LDL lipids was a prerequisite for any anti-inflammatory activity, lipid oxidation is no longer required in our experiments, suggesting that cationic lipids transport phospholipids through a different route and affect different pathways. This opens up new possibilities for manipulating inflammatory responses in gene therapy protocols but also in a general manner in immunological experiments.

Keywords. Cationic lipids, TNF-α, anti-inflammatory, phospholipids, CpG sequences.

Introduction

Cationic lipids are amphiphilic molecules capable of transporting nucleic acid or proteins into cells *in vitro* or *in vivo* [1–3]. The cellular pathway used by cationic lipid/DNA complexes (lipoplexes) to deliver DNA into the nucleus is still not satisfactorily understood even though endocytosis and endosomal escape are key steps of the transfection mechanism [4–6]. Intravenous injection of lipoplexes allows a good level of transfection in many organs, mainly in lung and kidneys [7–9]. Unfortunately, the interaction of some cationic lipids, or helper lipid with serum components, including lipoproteins, severely limits the transfection efficiency of these vectors [10–14]. Furthermore, intravenous injection of lipoplexes into mice induces pro-inflammatory cytokine secretion into the blood [15–17]. The immune response that limits the efficiency of these vectors has been attributed mainly to specific plasmid sequences, the so-called unmethylated CpG sequences, which are recognized by the Toll-like receptor 9 as a bacterial DNA [15, 18]. Although the mechanism of CpG sequences-induced inflammatory response is rather well understood nowadays, few or no solutions have been designed to circumvent this effect in gene therapy experiments [19, 20].

Our group has shown, in a murine model, that preinjecting DNA-free diC14-amidine liposomes

* Corresponding author.

[21–24] 1 h before lipoplex injection significantly reduces the lipoplex-induced tumor necrosis factor-α (TNF-α) secretion into the blood [25]. *In vitro*, free liposomes acquired this anti-inflammatory activity only when preincubated with serum [26]. Low-density lipoproteins (LDL) and triglyceride-rich lipoproteins were identified as the serum components that confer the anti-inflammatory activity to the diC14-amidine cationic liposomes [26].

In the present report, our data provide evidence that diC14-amidine confers new anti-inflammatory properties to phospholipids from LDL but also to synthetic phospholipids for which such an observation has not been previously reported. Whereas oxidation of LDL lipids was a prerequisite to any anti-inflammatory activity, in our experiments lipid oxidation was no longer required when associated with diC14-amidine, suggesting that cationic lipids transport phospholipids through a different route and affect different cellular pathways. This opens up new possibilities for manipulating inflammatory responses not only in gene therapy protocols, but also, in a general manner, in immunological experiments.

Materials and methods

Reagents. The unmethylated CpG oligodeoxynucleotides CpG ODN 1826 (5′-tcc atg acg ttc ctg acg tt-3′) (InvivoGen) used in this study were shown previously to have potent immunostimulatory effects on the murine immune system. 9S-hydroxy-10E,12Z-octadecadienoic acid [9-(S) HODE] is a Cayman Chemical product.

2-arachidonoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (PAPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2- distearoyl-glycero-3-phosphocholine (DSPC), 2-linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (PLPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) and 1,2-dimyristoyl-*sn*-glycero-3-phophoglycerol (DMPG) were purchased from Sigma-Aldrich. Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium (Rh-DHPE) was purchased from Invitrogen.

Cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) and 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol) were purchased from Avanti polar lipids. Dimethyldioctadecylammonium bromide (DDAB) was from Sigma-Aldrich and 3-tetradecylamino-*N*-tert-butyl-*N*′-tetradecyl-propionamidine (diC14-amidine) was synthesized as described [22].

Liposome preparation. Cationic lipids were dissolved in chloroform. After lipid film formation (by solvent evaporation under nitrogen stream), cationic lipids were resuspended in 10 mM HEPES and extruded seven times through a 0.4-μm polycarbonate filter at 55 °C as described previously [25]. Liposomes were then stored at 4 °C, and heated to 55 °C for 10 min just before an experiment. Same protocol was used for mixed liposomes except that phospholipid was added in chloroform to cationic lipid before formation of the lipid film.

Cell culture and incubation with diC14-amidine liposomes. RAW 264.7 macrophage cell line was cultured in DMEM (Invitrogen) supplemented by 5% FBS (BioWhittaker), 1 mM sodium pyruvate, 1 mM glutamine and antibiotics (Invitrogen) (complete medium).

RAW 264.7 cells were plated on 24-well plates at a density of 5×10^5 cells/well 24 h before experiments. diC14-amidine/lipid or mixed cationic lipids/phospholipids liposomes (in DMEM) were incubated with cells for 1 h. After this incubation, cells were washed twice with DMEM and stimulated with CpG ODN (0.5 μg/ml) for 4 h. Culture supernatants were collected and stored at −20 °C before TNF-α quantification by ELISA assay (R&D Systems). Proteins were quantified in the cell lysate using BCA (bicinchoninic acid) assay (Perbio) to normalize the TNF-α level.

Detection of several cytokines in cell supernatants by a Cytokine Array membrane was performed using RayBio® Mouse Cytokine Antibody Array II from RayBiotech and Kodak BioMax Chemiluminescence films following the manufacturers' instructions.

Isolation of serum lipids. Lipoproteins were isolated from human serum (from healthy volunteer) by KBr density gradient ultracentrifugation as described [27]. Briefly, a discontinuous KBr density gradient was prepared in ultracentrifugation tubes using KBr solutions as follows (from bottom to top): 2.5 ml of d = 1.24 g/ml,7.5 ml of d = 1.21 g/ml containing serum, 10 ml 1.125 g/ml, 10 ml 1.063 g/ml, 2.5 ml 1.019 g/ml, 2.5 ml 1.006 g/ml and 2.5 ml milli-Q water. Samples were then centrifuged at 105 000 *g* for 16 h at 4 °C. The fraction corresponding to LDL (density 1.028–1.050 g/ml) were sampled. KBr was removed from lipoproteins using gel filtration chromatography on PD-10 columns (Amersham Biosciences) and volumes of lipoprotein fractions were adjusted in HBS-20 buffer to obtain the serum concentration.

To extract lipids from lipoproteins, 250 μl of human serum lipoprotein solution was added to 3 ml meth-

anol, and vortexed before addition of 7 ml diethyl ether. The mixture was allowed to stand on ice for 10 min and centrifuged at 100 *g* for 5 min. The supernatant, containing lipids, was collected. Diethyl ether (10 ml) was added to the pellet, followed by incubation for 10 min on ice, and centrifugation at 100 *g* for 10 min to collect the supernatant. All supernatants were washed once by adding 3 ml methanol, and centrifuged as described before. The solvents were evaporated under a nitrogen stream and the resulting lipid film was hydrated with 250 µl HBS-20 buffer and vortexed for 1 min at 56 °C [28].

The lipid mixture extracted from lipoproteins was dispensed onto Silica Gel 60 TLC plates (Merck) and eluted twice using a hexane, diethyl-ether, acetic acid (80 : 20 : 2) mobile phase. Only a small part of the plate was revealed by iodine vapor to localize lipids on the plate. Separated lipids were scraped from the plate and dissolved in chloroform/methanol/water (5 : 5 : 1); the silica gel was removed by centrifugation. The solvent in the supernatant was evaporated. The resulting film was finally resuspended in HBS-10 at a seric concentration and vortexed at 56 °C for 1 min. DiC14-amidine/seric lipid mixed samples were prepared by incubating lipids extracted from LDLs with diC14-amidine liposomes (125 µg/ml diC14-amidine and lipids at half the concentration prevailing in the serum) for 30 min at 37 °C. After incubation, DMEM was added to obtain the final concentration of 50 µg/ml diC14-amidine.

Oxidation of PAPC. Oxidized PAPC was obtained by flushing filtered air on a lipid film for 72 h. The state of oxidation was controlled using mass spectrometry in positive mode as described by Watson et al. [29].

Uptake measurement. Liposomes (93 µM cationic lipid or 93 µM cationic lipid/PAPC or 93 µM PAPC in DMEM) were prepared as described under "Liposome preparation" except that 1 % Rh-DHPE was added for liposome formation. RAW 264.7 cells were incubated with these liposomes for 1 h then washed three times with DMEM and once with PBS buffer. Cells were lysed using 2 % SDS, 5 mM EDTA, 50 mM TRIS, 1 mM DTE, 1 mM PMSF, pH 8, the lysate was centrifuged for 15 min at 20 000 *g* and rhodamine fluorescence was quantified in the supernatant using a fluorimeter SLM-8000C. Percentage of uptake was obtained by comparison with the fluorescence emission of liposomes.

Lipid mixing assay. Fusion between diC14-amidine liposomes and PAPC liposomes was monitored using fluorescence resonance energy transfer assay (FRET) as described [12]. Cationic liposomes containing 1 % (molar ratio) *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) and Rh-PE were used to prepare liposomes as described above. PAPC liposomes were added to diC14-amidine at a molar ratio of 1 : 1 and loaded in a quartz fluorescence cell thermostated at 37 °C. The fluorescence was monitored using an SLM-8000 spectrofluometer with excitation and emission slits of 4 nm. Generally, samples were excited at 470 nm and emission of NBD was recorded at 535 nm (F_{NBD}). Control emission spectra were performed in parallel before and after fusion. The initial fluorescence of the labeled liposome suspension was recorded as 0 % fluorescence (F_0) and the 100 % fluorescence was determined after adding Triton X-100 at 0.1 % final concentration (F_{100}). Percentage of fusion was estimated from the fluorescence of NBD (at 535 nm): $\% F = (F_{NBD} - F_0)/(F_{100} - F_0)$.

Statistics. Unpaired two-tails *t*-test was used for statistical analysis.

Results

Phospholipids from lipoproteins are involved in TNF-α inhibition mediated by diC14-amidine liposomes. Components of a lipid extract from LDL were separated by thin layer chromatography (TLC). The different classes of lipids (cholesteryl ester, tri- di- and mono-glycerides, free fatty acids, cholesterol and phospholipids) were extracted from the thin layer and vortexed in buffer at the concentration prevailing in the serum. After 30 min incubation at 37 °C with diC14-amidine liposomes, the mixture of diC14-amidine and lipoproteic lipids was incubated with RAW 264.7 cells for 1 h. Cells were washed and finally stimulated with CpG oligonucleotides for 4 h and TNF-α secretion was quantified in the cell supernatant by ELISA assay (Fig. 1). Only phospholipids preincubated with diC14-amidine inhibited TNF-α secretion induced by CpG ODN. The other classes of lipids did not inhibit the TNF-α secretion.

Phospholipid oxidation is not required to inhibit TNF-α secretion. It has been reported that oxidized phospholipids from lipoproteins, mainly PAPC (1-palmitoyl 2-arachidonyl phosphatidylcholine) inhibit cytokine secretion induced by Toll-like receptors ligands, while non-oxidized phospholipids do not modify cytokine secretion [30–33]. Therefore, since PAPC is a major phospholipid component of lipoproteins, it can be argued that traces of oxidized PAPC are responsible for the anti-inflammatory property, independently of the cationic lipid added.

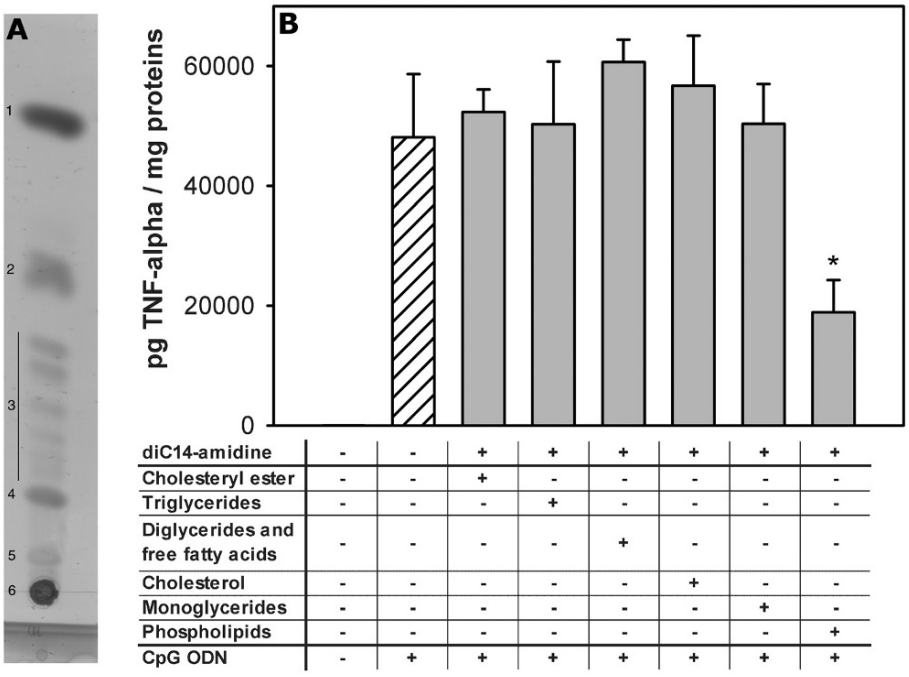


Figure 1. Phospholipids are responsible for the inhibitory effect of diC14-amidine liposomes on TNF-α secretion. (*A*) Thin layer chromatography of low-density lipoprotein (LDL) lipid extract eluted two times with hexane/diethylether/acetic acid 80 : 20 : 1. Spots: 1, cholesteryl ester; 2, triglycerides; 3, diglycerides and free fatty acids; 4, cholesterol; 5, monoglycerides; 6, phospholipids. (*B*) diC14-amidine liposomes were incubated with lipidic components for 30 min at 37 °C at a final concentration of 50 μg/ml in diC14-amidine. RAW 264.7 cells (5×10^5) were then incubated for 1 h with these suspensions (or medium for control), washed twice and stimulated with CpG ODN (0.5 μg/ml) for 4 h. Cell supernatants were then recovered and TNF-α was quantified by ELISA assay. Data represent means ± SD ($n = 3$) * $p < 0.05$ (*versus* CpG control).

To address this point, we compared the inhibitory effect of oxidized PAPC and of a mixed liposome made of diC14-amidine and PAPC.

As expected from the literature, control experiments showed that, in the absence of cationic lipid, oxidized PAPC, at a concentration of 300 μg/ml, inhibited CpG induced-TNF-α secretion on RAW 264.7 cells, whereas non-oxidized PAPC (at the same concentration) did not (Fig. 2A). At lower concentration (40 μg/ml), oxidized PAPC did not inhibit TNF-α secretion (Fig. 2A). In contrast, when cells were incubated for 1 h with mixed liposomes made of diC14-amidine and PAPC, oxidized or not, TNF-α secretion was inhibited (Fig. 2B). Interestingly, at a concentration at which oxidized PAPC alone did not affect TNF-α secretion (40 μg/ml), both oxidized and non-oxidized PAPC, mixed with diC14-amidine, caused a decrease of the CpG-induced TNF-α secretion (Fig. 2). Our experiments showed that oxidation of the phospholipid is not required to inhibit TNF-α secretion when it is used in combination with diC14-amidine. The association of PAPC with diC14-amidine allows an efficient inhibition of TNF-α at doses of PAPC lower than those used previously.

Incubation of RAW 264.7 cells with CpG sequences induces the secretion of several cytokines in the supernatant (Fig. 3B) as compared to the cytokine secretion into control cells supernatant (Fig. 3A). When cells were incubated with diC14-amidine/PAPC prior CpG stimulation, inhibition of secretion was observed mainly for interleukin (IL)-6, IL-12, RANTES (regulated upon activation, normal T cell expressed, and secreted) and TNF-α, while other cytokines were not affected [monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 α (MIP-1α), MIP-2, etc.] (Fig. 3C).

We compared the cytokine secretion profile obtained with diC14-amidine/PAPC liposomes to the profile obtained with 9-HODE, a peroxisome proliferator activated receptor (PPAR)-γ ligand with anti-inflammatory properties (Fig. 3C and D). The similar inhibition profiles suggests that diC14-amidine/PAPC liposomes may use a similar mode of action (Fig. 3).

Both saturated and unsaturated phospholipids inhibit TNF-α secretion when mixed with diC14-amidine. Since PAPC is a major phospholipid of lipoproteins, we wondered whether the inhibitory effect was attributable to PAPC alone or if other phospholipids, even those not present in serum, would also inhibit TNF-α secretion in association with diC14-amidine. The requirement for unsaturated acyl chains was also examined.

Liposomes made of diC14-amidine and unsaturated (PLPC, POPC, PAPC) or saturated (DPPC, DMPC, DSPC) phosphatidylcholines were incubated for 1 h with cells before activation with CpG sequences for 4 h (Fig. 4A). All phosphatidylcholines, when mixed with diC14-amidine, prevented TNF-α secretion induced by CpG ODN. The same lipids, at the same concentrations, in the absence of diC14-amidine revealed only a minor effect on TNF-α secretion (Fig. 4B). Therefore, phospholipids not present in

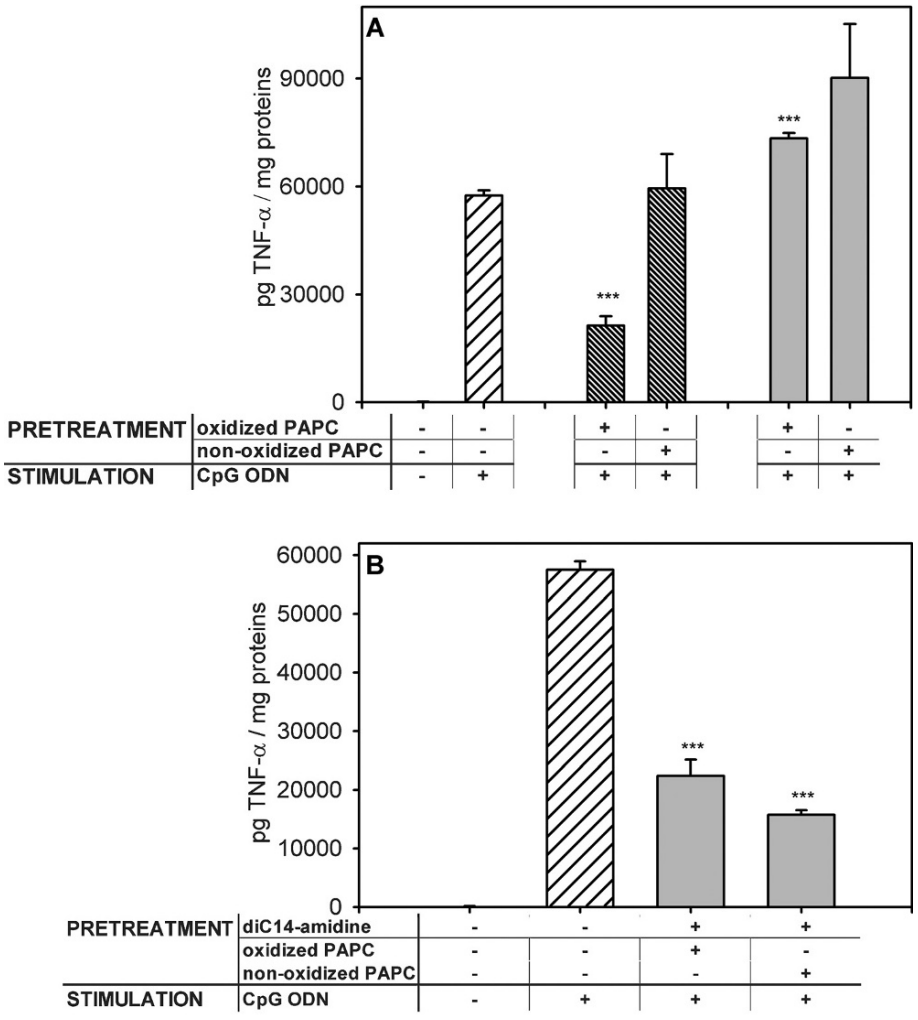


Figure 2. (*A*) Inhibition of the CpG-induced TNF-α secretion by oxidized PAPC. RAW 264.7 (5×10^5 cells/well) cells were incubated for 1 h with medium or with PAPC (300 μg/ml for dark gray, 40 μg/ml for dashed lines) oxidized or not. After this incubation, cells were washed twice and stimulated with CpG ODN (0.5 μg/ml) for 4 h. Cell supernatants were then recovered and TNF-α was quantified by ELISA assay. Data represent means ± SD ($n = 3$) *** $p < 0.001$ (*versus* CpG control). (*B*) Inhibition of the CpG-induced TNF-α secretion by oxidized and non-oxidized PAPC, in the presence of diC14-amidine. RAW 264.7 cells (5×10^5 cells/well) were incubated for 1 h with medium or with diC14-amidine/PAPC (40 μg/ml diC14-amidine, 40 μg/ml PAPC). After this incubation, cells were washed and stimulated with CpG ODN (0.5 μg/ml) for 4 h. Cell supernatants were then recovered and TNF-α was quantified by ELISA assay. Data represent means ± SD ($n = 3$) * $p < 0.05$; *** $p > 0.01$ (*versus* CpG control).

lipoproteins also show a preventing effect on the TNF-α secretion provided they are associated with diC14-amidine. Interestingly, the nature of the lipid acyl chain did not seem to influence the inhibitory role of diC14-amidine/phosphatidylcholine liposomes (Fig. 4A) and even saturated lipids have an inhibitory effect, confirming that oxidation of the phospholipid is not required.

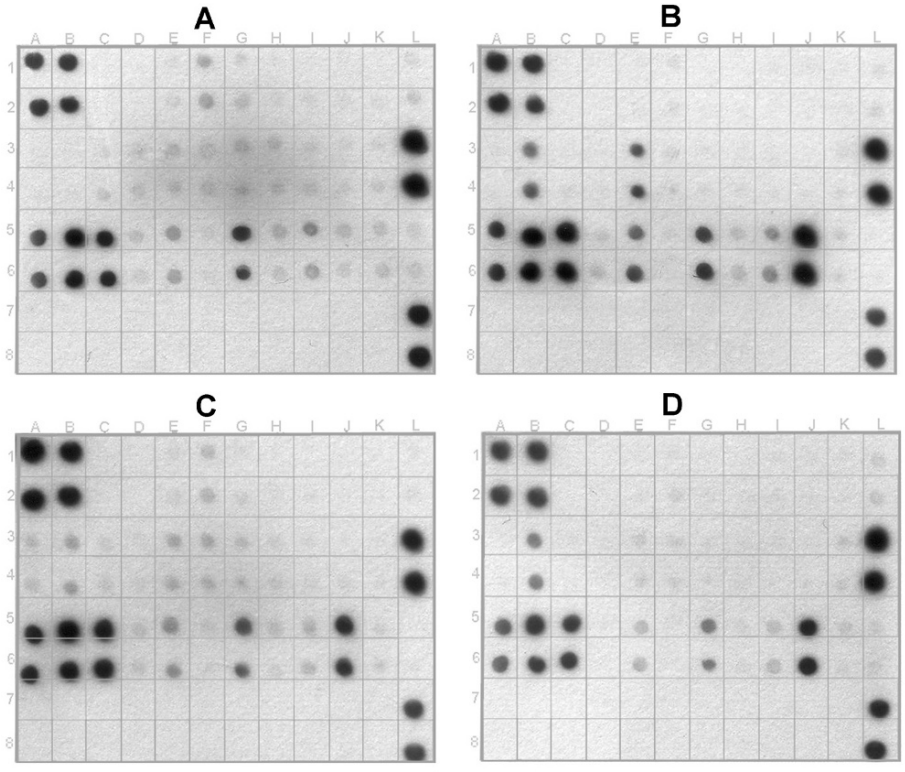
Other synthetic phospholipids (DMPE, DPPS, DMPG) were also tested using the same protocol (Fig. 4C). All of them inhibited TNF-α secretion, in association with diC14-amidine and the sequence of inhibition efficiency was as follows: phosphatidylcholine > phosphatidylethanolamine > phosphatidylserine > phosphatidylglycerol.

Is the inhibitory effect on TNF-α secretion specific to diC14-amidine? We have compared the effect of different commercially available cationic lipids, in combination with phospholipids, on TNF-α secretion. Each cationic lipid (DOTAP, DC-Chol, DDAB) was prepared as a mixed liposome (molar ratio 1 : 1) with a phospholipid extract from LDL and incubated with cells. TNF-α secretion induced by CpG ODN was finally quantified in cell supernatants (Fig. 5). Among these cationic lipids, only diC14-amidine significantly inhibited the TNF-α secretion induced by CpG sequences.

This peculiar effect did not depend on the capture efficiency of the liposomes. Indeed, all cationic lipids tested improved the lipid uptake to the same extent (Fig. 6), suggesting that diC14-amidine possess a specific property allowing it to prevent CpG-induced cytokine secretion when mixed with phospholipids. This aspect is discussed further.

Discussion

We have demonstrated that cationic lipids do confer an anti-inflammatory activity to phospholipids. Anti-inflammatory activity of several specific phospholi-



E

	A	B	C	D	E	F	G	H	I	J	K	L
1	Pos	Pos	Neg	Neg	6Ckine	CTACK	Eotaxin	GCSF	GM-CSF	IL-2	IL-3	IL-4
2	Pos	Pos	Neg	Neg	6Ckine	CTACK	Eotaxin	GCSF	GM-CSF	IL-2	IL-3	IL-4
3	IL-5	IL-6	IL-9	IL-10	IL-12p40	IL12-p70	IL-13	IL-17	IFN-γ	KC	Leptin	MCP-1
4	IL-5	IL-6	IL-9	IL-10	IL-12p40	IL12-p70	IL-13	IL-17	IFN- γ	KC	Leptin	MCP-1
5	MCP-5	MIP-1a	MIP-2	MIP-3	RANTES	SCF	sTNFri	TARC	TIMP-1	TNF-α	Tpo	VEGF
6	MCP-5	MIP-1a	MIP-2	MIP-3	RANTES	SCF	sTNFri	TARC	TIMP-1	TNF-α	Tpo	VEGF
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Pos
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Pos

Figure 3. Inhibition of the CpG-induced cytokine secretion by diC14-amidine/PAPC. RAW 264.7 (5×10^5 cells/well) cells were incubated for 1 h with medium (*A*, *B*), with diC14-amidine/PAPC (93 μM) (*C*) or 9-(S) HODE (25 μg/ml) (*D*). After this incubation, cells were washed twice and stimulated with CpG ODN (0.5 μg/ml) for 4 h (for *B*–*D*). Cell supernatants were then recovered and cytokine secretion was detected by chemiluminescence using RayBio® Mouse Cytokine Antibody Array II from RayBiotech. By comparing the signal intensities, relative expression levels of cytokines can be made. (*E*) Map for RayBio® Mouse Cytokine Antibody Array II detection.

pids has been reported previously. Phosphatidylcholines from oxidized LDLs (mainly oxidized PAPC) inhibit cytokine secretion induced by lipopolysaccharides (LPSs) or CpG sequences [30–32]. The mechanism of action is largely unknown, although several studies proposed a direct interaction between LPS receptors (CD14, TLR4 or LBP) and oxidized phospholipids, decreasing the binding of LPS to these receptors [31, 34, 35]. Another possibility is that some signaling pathways implicated in immune responses are affected downstream of the receptor, since the activity of several transactivating factors is modified by oxidized phospholipids (e.g., NF-κB, p38, CREB, PPARs) [31, 36, 37].

Oxidation of these phospholipids is required to obtain the anti-inflammatory activity and their specific bioactivity seems to depend mainly on the acyl chain in the *sn-2* position of the phospholipid (oxidized acyl chain) and less on the second acyl chain or the polar headgroup [32].

Anti-inflammatory properties have also been described for a few non-oxidized phospholipids. A schistosome-specific lysophosphatidylserine (constituted of long unsaturated alkyl chain) is able to induce a down-modulation of immune responses *via* the activation of TLR2 and the development of regulatory T cells [38]. Phosphatidylglycerol from *Treponema* (a phosphatidylglycerol with a vinyl ether group instead of an acyl group) has been shown to inhibit cytokine secretion and NF-κB activation induced by LPS [39]. However, this effect could be reproduced by using "conventional" phosphatidylglycerols with at least one unsaturated chain (POPG and DOPG) [39, 40]. This property has been attributed to an inhibitory effect of phospholipids on LPS recognition by one of its receptors (CD14) [39, 40]. This conclusion should be broadened to other receptors, at least the CpG receptor (TLR9) to account for our results.

Some mammalian phospholipids also exert an anti-inflammatory property. Phosphatidylserine liposomes

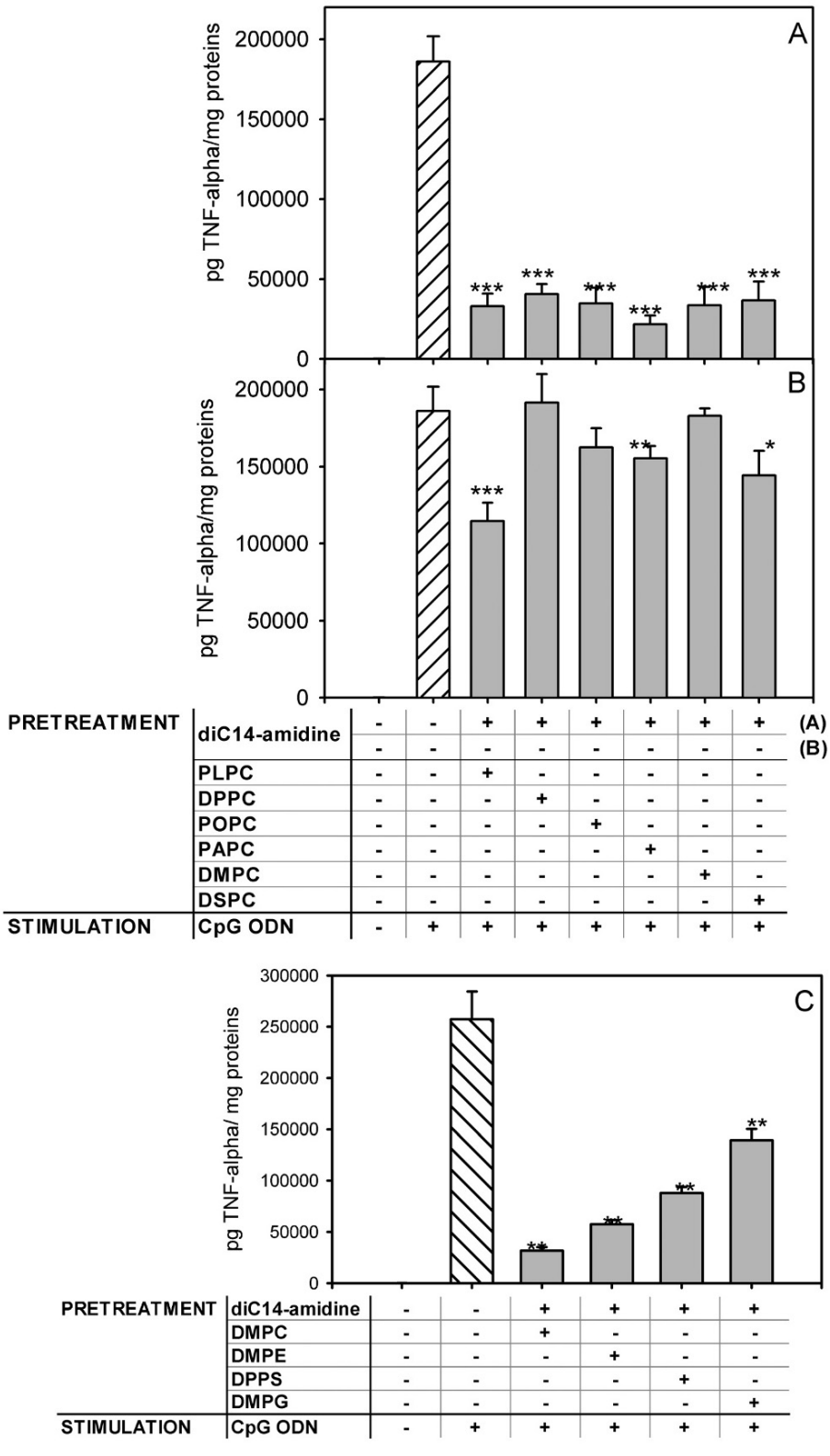


Figure 4. Synthetic phospholipid/diC14-amidine mixed liposomes inhibit the secretion of TNF-α by murine macrophages. (*A*, *C*) RAW 264.7 cells (5×10^5 cells/well) were incubated for 1 h with medium or mixed liposomes of diC14-amidine/phospholipid (molar ratio 1:1, 50 μg/ml 93 μM diC14-amidine), then washed and stimulated with CpG ODN (0.5 μg/ml) for 4 h. After this incubation, cell supernatants were recovered and TNF-α was quantified by ELISA assay. Data represent means ± SD ($n = 3$) *** $p < 0.001$, ** $p < 0.01$ (*versus* CpG control). (*B*) RAW 264.7 cells (5×10^5 cells/well) were incubated for 1 h with medium or phosphatidylcholine liposomes (93 μM), then washed and stimulated with CpG ODN (0.5 μg/ml) for 4 h. After this incubation, cell supernatants were recovered and TNF-α was quantified by ELISA assay. Data represent means ± SD ($n = 3$) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (*versus* CpG control).

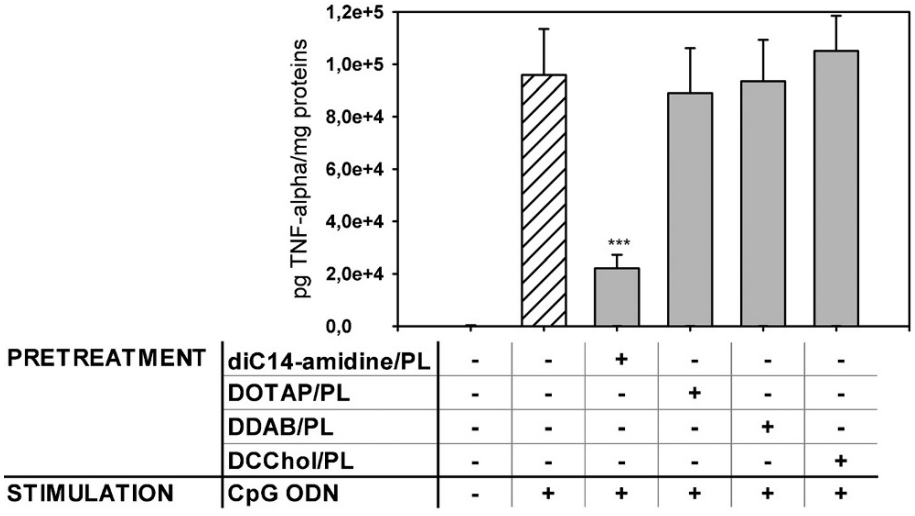


Figure 5. Inhibitory property of different cationic lipids on TNF-α secretion. RAW 264.7 cells (5×10^5 cells/well) were incubated with medium or with mixed liposomes of cationic lipid/phospholipid (molar ratio 1:1, 93 μM cationic lipid) for 1 h. After this incubation, cells were washed twice and stimulated with CpG ODN (0.5 μg/ml) for 4 h. After this incubation, cell supernatants were recovered and TNF-α was quantified by ELISA assay. Data represent means ± SD ($n = 3$) *** $p < 0.001$ (*versus* CpG control).

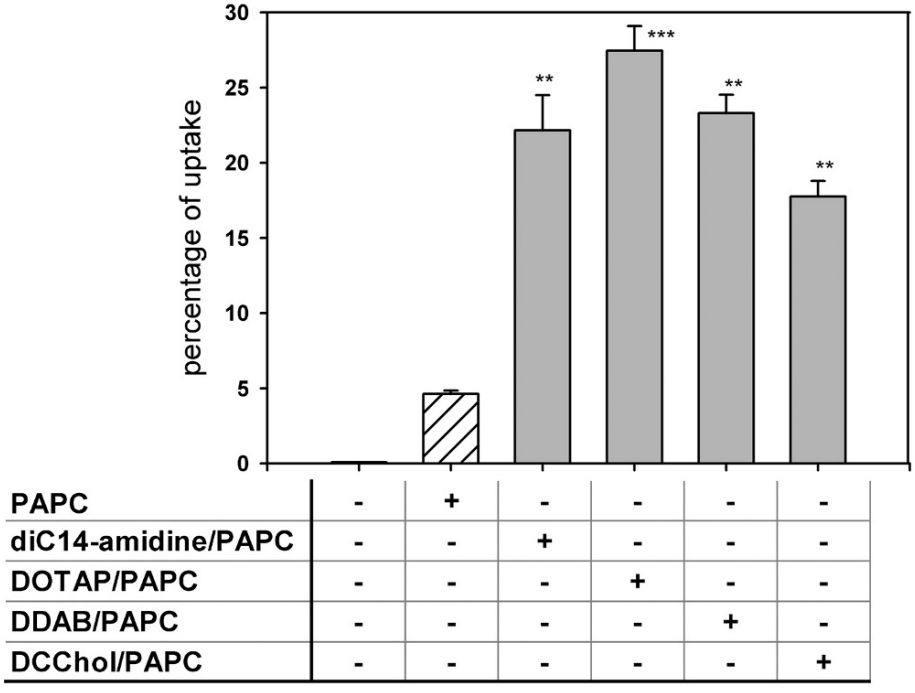


Figure 6. Uptake of phospholipid-cationic lipid mixtures by cells. PAPC liposomes (93 μM) or mixed liposomes of cationic lipid/PAPC (93 μM, molar ratio 1:1), labeled with 1% Rh-DHPE were incubated with 5×10^5 RAW 264.7 cells for 1 h. After this incubation, cells were washed three times with phosphate buffer and lysed. Fluorescence of the cell lysate was quantified using a fluorimeter and normalized with the fluorescence of liposomal preparation before the incubation.

and apoptotic cells presenting phosphatidylserine on their extracytoplasmic side were reported to induce the development of T regulatory cells [41, 42]. Pulmonary surfactant, characterized by a high content of phospholipids plays also a role in the modulation of inflammatory response, inhibiting cytokine release and oxidative response in lung leukocytes stimulated with LPS and this effect was attributed to DPPC [43] or DOPG [40]. All the "anti-inflammatory" phospholipids (except DPPC) described in the literature were unsaturated, and the nature of the lipid acyl chains seemed to be crucial for their ability to inhibit the immune response. The range of concentration used in these works was generally high (100–500 μg/ml phospholipids).

All the phospholipids tested in the present work inhibited the CpG-induced TNF-α secretion at lower concentrations (40–80 μg/ml) (Fig. 4A and C). Neither the polar head group, the oxidation state, nor the length or the acyl chain saturation state of the phospholipid influenced the inhibition of TNF-α secretion by diC14-amidine/phospholipids. It is worth mentioning that phospholipids alone, at the same concentration, did not inhibit the TNF-α release by cells (Fig. 4B). Consequently, diC14-amidine confers an anti-TNF-α secretion activity to phospholipids that are not anti-inflammatory by themselves (saturated lipids, non-oxidized). We have also demonstrated that mixed liposomes made

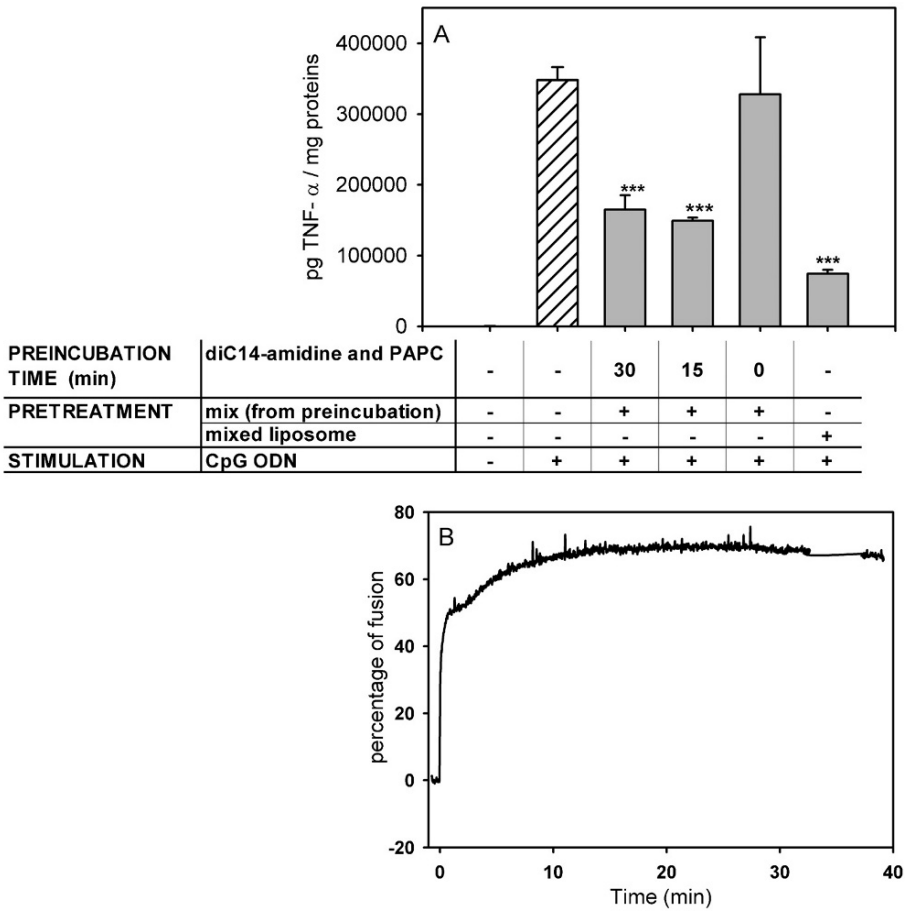


Figure 7. Effect of the phospholipid and diC14-amidine preincubation. (*A*) DiC14-amidine liposomes were incubated with PAPC liposomes for 0, 15 or 30 min at 37 °C to reach a final concentration of 50 μg/ml diC14-amidine and 72 μg/ml PAPC. Mixed liposomes of diC14-amidine/PAPC (molar ratio 1:1, 50 μg/ml in diC14-amidine) were also prepared. RAW 264.7 cells (5×10^5) were then incubated for 1 h with these lipid mixtures (or medium for control), then washed twice and stimulated with CpG ODN (0.5 μg/ml) for 4 h. Cell supernatants were then recovered and TNF-α was quantified by ELISA assay. Data represent means ± SD ($n = 3$) * $p < 0.05$ (*versus* CpG control). (*B*) Time course of lipid mixing between diC14-amidine liposomes and PAPC liposomes. PAPC liposomes were added to labeled diC14-amidine liposomes (containing 1% of Rh-PE and NBD-PE) into a quartz cell at molar ratio of 1:1 and fluorescence of NBD-PE was recorded (excitation: 470 nm; emission: 535 nm) at different times as a measure of the extent of lipid mixing.

of diC14-amidine and PAPC could inhibit the secretion of several cytokines, including IL-6, IL-12, RANTES and TNF-α (Fig. 3C).

The fact that mixed liposomes affect other cytokines secretion suggests a more general effect of these lipids on the inflammatory reaction. This result also demonstrates that the inhibitory effect of diC14-amidine/PAPC on cytokine secretion is not related to a global nonspecific protein synthesis/secretion inhibition since secretion of several cytokines (MCP-1, MIP-1α, MIP-2) was not affected (Fig. 3C).

Experiments carried out with different preincubation times (0, 15, 30 min) between liposomes of PAPC and diC14-amidine liposomes demonstrated that contact between cationic and non-cationic lipids before incubation with cells was a prerequisite to any inhibitory effect on TNF-α secretion (Fig. 7A). FRET experiments demonstrated spontaneous lipid mixing between diC14-amidine liposomes and phospholipid liposomes within a few minutes (Fig. 7B), suggesting that fusion between cationic liposomes and liposomes made of neutral phospholipids spontaneously occurs and that intimate mixing of lipids is required for their anti-inflammatory property. Consistent with this observation, only liposomes made of a mixture of diC14-amidine and phospholipid showed an efficient inhibition (Fig. 7A).

Our data show that the presence of the two lipids (cationic lipid and phospholipid) inside the same entity is required to inhibit the CpG-induced TNF-α secretion. This might suggest a mechanism in which the cationic lipid is recognized by a receptor at the plasma membrane, while another receptor, in the vicinity of the first one, would recognize the phospholipid. The association between these two receptors, after their co-recognition by ligands would be required for inhibition of the immune response.

DiC14-amidine enhances significantly the phospholipid cell uptake (Fig. 6). However, the percentage of uptake into cells after 1 h of incubation was identical for all cationic lipids tested (DOTAP, DDAB, DC-Chol) whatever their anti-inflammatory activity. Clearly, there is no improvement of phospholipid uptake that could explain the inhibitory property of diC14-amidine/phospholipid.

Since diC14-amidine possesses a fast fusion activity (lipid mixing) that is not shared by all cationic lipids [12], the fusogenic property of diC14-amidine liposomes may be crucial for the mechanism of TNF-α inhibition. A fusion step with the plasma membrane

and/or the endosomal membrane may be required for a targeted delivery of the phospholipid into the cell as illustrated by recent FRET experiments revealing a lipid mixing between labeled liposomes and macrophages (preliminary data not shown). As transport by cationic liposomes in the proximity of the nucleus, *via* interaction with the endoplasmic reticulum has been suggested by other groups [44–46], it is possible that fusogenic cationic lipids, like diC14-amidine, could fuse with various intracellular membranes, including those of mitochondria, endoplasmic reticulum and nucleus. Preliminary fluorescent confocal microscopy experiments confirmed that, in contrast to DOTAP, diC14-amidine liposomes fuse quickly with intracellular membranes, including membranes surrounding the nucleus (data not shown). This effect could contribute to transport phospholipids near/into nuclear membranes, allowing an efficient interaction with nuclear receptors, including PPARs. These receptors are located in the nuclear periphery and were described for their interaction with phospholipids and their possible role in the anti-inflammatory process [47, 48].

Our experiments on cytokine secretion by RAW 264.7 cells revealed strong similarities between the pattern induced by diC14-amidine/PAPC and the pattern obtained with 9-HODE, a PPAR-γ ligand (Fig. 3), which support the idea of an interaction with a PPAR. A more extended description of the pathway involved in this property is under progress.

In summary, our results demonstrate that diC14-amidine has the potency to interact with serum components (lipids and proteins) and to transport them inside the cells. We have taken advantage of this property of diC14-amidine to mediate phospholipid transport inside the cell and to generate an anti-inflammatory response. This opens the way to an evaluation of the anti-inflammatory properties of a large number of phospholipids. This work also suggests that, whereas most studies focus on the improvement of the transfection efficiency of cationic lipids, the gene transporter itself should be considered as a potential modifying agent for the cell physiology.

Beside their own activity, cationic lipids might transport exogenous molecules (like serum components) into cell compartments that are poorly accessible under normal circumstances and affect other cellular properties. This could lead to the discovery of new pharmacological activities for substances that have no access to intracellular targets by themselves and concomitantly to the development of new drugs.

Acknowledgements. This work was supported in part by the Walloon Region of Belgium, Direction Generales des Technologies, de la Recherche et de l'Energie (C. Lonez). M. Vandenbranden is a Research Associate of the Belgian National Fund for Scientific Research. A. Legat is a F.R.I.A. fellow (Fonds de la Recherche Scientifique pour l'Industrie et l'Agriculture).

1 Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987) Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA 84, 7413–7417.
2 Martin, B., Sainlos, M., Aissaoui, A., Oudrhiri, N., Hauchecorne, M., Vigneron, J. P., Lehn, J. M. and Lehn, P. (2005) The design of cationic lipids for gene delivery. Curr. Pharm. Des. 11, 375–394.
3 Karmali, P. P. and Chaudhuri, A. (2006) Cationic liposomes as non-viral carriers of gene medicines: Resolved issues, open questions, and future promises. Med. Res. Rev. 27, 696–722.
4 Elouahabi, A. and Ruysschaert, J. M. (2005) Formation and intracellular trafficking of lipoplexes and polyplexes. Mol. Ther. 11, 336–347.
5 Zuhorn, I. S., Engberts, J. B. and Hoekstra, D. (2007) Gene delivery by cationic lipid vectors: Overcoming cellular barriers. Eur. Biophys. J. 36, 349–362.
6 Wasungu, L. and Hoekstra, D. (2006) Cationic lipids, lipoplexes and intracellular delivery of genes. J. Control. Release 116, 255–264.
7 Liu, F., Qi, H., Huang, L. and Liu, D. (1997) Factors controlling the efficiency of cationic lipid-mediated transfection *in vivo via* intravenous administration. Gene Ther. 4, 517–523.
8 Templeton, N. S., Lasic, D. D., Frederik, P. M., Strey, H. H., Roberts, D. D. and Pavlakis, G. N. (1997) Improved DNA: Liposome complexes for increased systemic delivery and gene expression. Nat. Biotechnol. 15, 647–652.
9 McLean, J. W., Fox, E. A., Baluk, P., Bolton, P. B., Haskell, A., Pearlman, R., Thurston, G., Umemoto, E. Y. and McDonald, D. M. (1997) Organ-specific endothelial cell uptake of cationic liposome-DNA complexes in mice. Am. J. Physiol. 273, H387–H404.
10 Escriou, V., Ciolina, C., Lacroix, F., Byk, G., Scherman, D. and Wils, P. (1998) Cationic lipid-mediated gene transfer: Effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes. Biochim. Biophys. Acta 1368, 276–288.
11 Li, S., Tseng, W. C., Stolz, D. B., Wu, S. P., Watkins, S. C. and Huang, L. (1999) Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: Implications for intravenous lipofection. Gene Ther. 6, 585–594.
12 Tandia, B. M., Lonez, C., Vandenbranden, M., Ruysschaert, J. M. and Elouahabi, A. (2005) Lipid mixing between lipoplexes and plasma lipoproteins is a major barrier for intravenous transfection mediated by cationic lipids. J. Biol. Chem. 280, 12255–12261.
13 Tandia, B. M., Vandenbranden, M., Wattiez, R., Lakhdar, Z., Ruysschaert, J. M. and Elouahabi A. (2003) Identification of human plasma proteins that bind to cationic lipid/DNA complex and analysis of their effects on transfection efficiency: Implications for intravenous gene transfer. Mol. Ther. 8, 264–273.
14 Son, K. K., Patel, D. H., Tkach, D. and Park, A. (2000) Cationic liposome and plasmid DNA complexes formed in serum-free medium under optimum transfection condition are negatively charged. Biochim. Biophys. Acta 1466, 11–15.
15 Li, S., Wu, S. P., Whitmore, M., Loeffert, E. J., Wang, L., Watkins, S. C., Pitt, B. R. and Huang, L. (1999) Effect of immune response on gene transfer to the lung *via* systemic administration of cationic lipidic vectors. Am. J. Physiol. 276, L796–804.
16 Dow, S. W., Fradkin, L. G., Liggitt, D. H., Willson, A. P., Heath, T. D. and Potter, T. A. (1999) Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. J. Immunol. 163, 1552–1561.

17 Zhao, H., Hemmi, H., Akira, S., Cheng, S. H., Scheule, R. K. and Yew, N. S. (2004) Contribution of Toll-like receptor 9 signaling to the acute inflammatory response to nonviral vectors. Mol. Ther. 9, 241–248.
18 Yew, N. S., Wang, K. X., Przybylska, M., Bagley, R. G., Stedman, M., Marshall, J., Scheule, R. K. and Cheng, S. H. (1999) Contribution of plasmid DNA to inflammation in the lung after administration of cationic lipid:pDNA complexes. Hum Gene Ther. 10, 223–234.
19 Zhang, J. S., Liu, F. and Huang, L. (2005) Implications of pharmacokinetic behavior of lipoplex for its inflammatory toxicity. Adv. Drug Deliv. Rev. 57, 689–698.
20 Liu, F., Shollenberger, L. M. and Huang, L. (2004) Non-immunostimulatory nonviral vectors. FASEB J. 18, 1779–1781.
21 Defrise-Quertain, F., Duquenoy, P., Brasseur, R., Brak, P., Caillaux, B., Fuks, R. and Ruysschaert, J. M. (1986) Vesicle formation by double long-chain amidines. J. Chem. Soc. Chem. Commun. 14, 1060–1062.
22 Ruysschaert, J. M., el Ouahabi, A., Willeaume, V., Huez, G., Fuks, R., Vandenbranden, M. and Di Stefano, P. (1994) A novel cationic amphiphile for transfection of mammalian cells. Biochem. Biophys. Res. Commun. 203, 1622–1628.
23 Pector, V., Backmann, J., Maes, D., Vandenbranden, M. and Ruysschaert, J. M. (2000) Biophysical and structural properties of DNA. diC(14)-amidine complexes. Influence of the DNA/lipid ratio. J. Biol. Chem. 275, 29533–29538.
24 Pector, V., Caspers, J., Banerjee, S., Deriemaeker, L., Fuks, R., El Ouahabi, A., Vandenbranden, M., Finsy, R. and Ruysschaert, J. M. (1998) Physico-chemical characterization of a double long-chain cationic amphiphile (Vectamidine) by microelectrophoresis. Biochim. Biophys. Acta 1372, 339–346.
25 Elouahabi, A., Flamand, V., Ozkan, S., Paulart, F., Vandenbranden, M., Goldman, M. and Ruysschaert, J. M. (2003) Free cationic liposomes inhibit the inflammatory response to cationic lipid-DNA complex injected intravenously and enhance its transfection efficiency. Mol. Ther. 7, 81–88.
26 Lonez, C., Vandenbranden, M., Ouali, M., Legat, A., Ruysschaert, J. M. and Elouahabi, A. (2006) Free diC14-amidine liposomes inhibit the TNF-alpha secretion induced by CpG sequences and lipopolysaccharides: Role of lipoproteins. Mol. Membr. Biol. 23, 227–234.
27 Chapman, M. J., Goldstein, S., Lagrange, D. and Laplaud, P. M. (1981) A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. J. Lipid Res. 22, 339–358.
28 Osborne, J. C. Jr. (1986) Delipidation of plasma lipoproteins. Methods Enzymol. 128, 213–222.
29 Watson, A. D., Leitinger, N., Navab, M., Faull, K. F., Horkko, S., Witztum, J. L., Palinski, W., Schwenke, D., Salomon, R. G., Sha, W., Subbanagounder, G., Fogelman, A. M. and Berliner, J. A. (1997) Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence *in vivo*. J. Biol. Chem. 272, 13597–13607.
30 Leitinger, N., Tyner, T. R., Oslund, L., Rizza, C., Subbanagounder, G., Lee, H., Shih, P. T., Mackman, N., Tigyi, G., Territo, M. C., Berliner, J. A. and Vora, D. K. (1999) Structurally similar oxidized phospholipids differentially regulate endothelial binding of monocytes and neutrophils. Proc. Natl. Acad. Sci. USA 96, 12010–12015.
31 Bochkov, V. N., Mechtcheriakova, D., Lucerna, M., Huber, J., Malli, R., Graier, W. F., Hofer, E., Binder, B. R. and Leitinger, N. (2002) Oxidized phospholipids stimulate tissue factor expression in human endothelial cells *via* activation of ERK/EGR-1 and Ca(++)/NFAT. Blood 99, 199–206.
32 Subbanagounder, G., Leitinger, N., Schwenke, D. C., Wong, J. W., Lee, H., Rizza, C., Watson, A. D., Faull, K. F., Fogelman, A. M. and Berliner, J. A. (2000) Determinants of bioactivity of oxidized phospholipids. Specific oxidized fatty acyl groups at the sn-2 position. Arterioscler. Thromb. Vasc. Biol. 20, 2248–2254.
33 Ma, Z., Li, J., Yang, L., Mu, Y., Xie, W., Pitt, B. and Li, S. (2004) Inhibition of LPS- and CpG DNA-induced TNF-alpha response by oxidized phospholipids. Am. J. Physiol. Lung Cell. Mol. Physiol. 286, L808–L816.
34 Walton, K. A., Gugiu, B. G., Thomas, M., Basseri, R. J., Eliav, D. R., Salomon, R. G. and Berliner, J. A. (2006) A role for neutral sphingomyelinase activation in the inhibition of LPS action by phospholipid oxidation products. J. Lipid Res. 47, 1967–1974.
35 Miller, Y. I., Chang, M. K., Binder, C. J., Shaw, P. X. and Witztum, J. L. (2003) Oxidized low density lipoprotein and innate immune receptors. Curr. Opin. Lipidol. 14, 437–445.
36 Davies, S. S., Pontsler, A. V., Marathe, G. K., Harrison, K. A., Murphy, R. C., Hinshaw, J. C., Prestwich, G. D., Hilaire, A. S., Prescott, S. M., Zimmerman, G. A. and McIntyre, T. M. (2001) Oxidized alkyl phospholipids are specific, high affinity peroxisome proliferator-activated receptor gamma ligands and agonists. J. Biol. Chem. 276, 16015–16023.
37 Birukov, K. G., Leitinger, N., Bochkov, V. N. and Garcia, J. G. (2004) Signal transduction pathways activated in human pulmonary endothelial cells by OxPAPC, a bioactive component of oxidized lipoproteins. Microvasc. Res. 67, 18–28.
38 van der Kleij, D., Latz, E., Brouwers, J. F., Kruize, Y. C., Schmitz, M., Kurt-Jones, E. A., Espevik, T., de Jong, E. C., Kapsenberg, M. L., Golenbock, D. T., Tielens A. G. and Yazdanbakhsh, M. (2002) A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. J. Biol. Chem. 277, 48122–48129.
39 Hashimoto, M., Asai, Y. and Ogawa, T. (2003) Treponemal phospholipids inhibit innate immune responses induced by pathogen-associated molecular patterns. J. Biol. Chem. 278, 44205–44213.
40 Wu, Y. Z., Medjane, S., Chabot, S., Kubrusly, F. S., Raw, I., Chignard, M. and Touqui, L. (2003) Surfactant protein-A and phosphatidylglycerol suppress type IIA phospholipase A2 synthesis *via* nuclear factor-kappaB. Am. J. Respir. Crit. Care Med. 168, 692–699.
41 Otsuka, M., Negishi, Y. and Aramaki, Y. (2007) Involvement of phosphatidylinositol-3-kinase and ERK pathways in the production of TGF-beta1 by macrophages treated with liposomes composed of phosphatidylserine. FEBS Lett. 581, 325–330.
42 Huynh, M. L., Fadok, V. A. and Henson, P. M. (2002) Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. J. Clin. Invest. 109, 41–50.
43 Tonks, A., Morris, R. H., Price, A. J., Thomas, A. W., Jones, K. P. and Jackson, S. K. (2001) Dipalmitoylphosphatidylcholine modulates inflammatory functions of monocytic cells independently of mitogen activated protein kinases. Clin. Exp. Immunol. 124, 86–94.
44 Kamiya, H., Fujimura, Y., Matsuoka, I. and Harashima, H. (2002) Visualization of intracellular trafficking of exogenous DNA delivered by cationic liposomes. Biochem. Biophys. Res. Commun. 298, 591–597.
45 Cao, A., Briane, D., Coudert, R., Vassy, J., Lievre, N., Olsman, E., Tamboise, E., Salzmann, J. L., Rigaut, J. P. and Taillandier, E. (2000) Delivery and pathway in MCF7 cells of DNA vectorized by cationic liposomes derived from cholesterol. Antisense Nucleic Acid Drug Dev. 10, 369–380.
46 Friend, D. S., Papahadjopoulos, D. and Debs, R. J. (1996) Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. Biochim. Biophys. Acta 1278, 41–50.
47 Daynes, R. A. and Jones, D. C. (2002) Emerging roles of PPARs in inflammation and immunity. Nat. Rev. Immunol. 2, 748–759.
48 Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H. and Evans, R. M. (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell 93, 229–240.