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European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 817–823
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Cationic lipid/DNA complexes induce TNF-α secretion in splenic macrophages

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Received 23 October 2007; accepted in revised form 31 January 2008
Available online 9 February 2008

Abstract

Cationic lipids are widely used as vectors to deliver DNA into mammalian cells *in vitro* and *in vivo*. However, cationic lipid/DNA lipoplexes induce an inflammatory response, characterized by pro-inflammatory cytokine secretion, which severely limits their use. The main goal of this work is to identify the organs and the cell type involved in TNF-α secretion after lipoplex injection. We determined the kinetics of distribution of the cationic lipid/DNA complex in blood, lung, liver and spleen and quantified the TNF-α amount in organ homogenates and in the serum at different points of times. Increase in TNF-α production was only observed in the spleen and no significant increase of TNF-α production could be observed in the other organs. Fractionation of spleen cells revealed that macrophages were mainly responsible for TNF-α secretion. This observation was verified *in vivo* by using macrophage-removing agents. In conclusion, we show here that the TNF-α secreted in the serum after intravenous injection of lipoplexes comes mainly from the splenic macrophages.

Keywords: Cationic liposomes; Lipoplexes; Macrophage; DiC14-amidine; Spleen; Transfection

1. Introduction

Cationic lipids have been widely used as vectors to deliver DNA into mammalian cells, both *in vitro* and *in vivo*, and may represent a safe alternative to viral vectors for gene therapy applications [1–4]. Systemic administration of lipoplexes in animal models results in reporter gene expression in several organs, including lung, liver, heart, kidney and spleen [2–4]. The lung shows the highest gene expression, and endothelial cells are the main transfected cells [5,6].

However, intravenously-injected cationic lipid–DNA lipoplexes induce an acute inflammatory response characterized by cytokine (TNF-α, IFN-γ) release in the serum that limits the use of these vectors and is attributed mainly to the plasmid CpG sequences, recognized by Toll-like receptors of immune cells [7,8]. Since intravenous injection of cationic liposomes or DNA alone failed to induce an inflammatory response, it was suggested that complexation of DNA with cationic liposomes strongly potentiated the immunostimulatory effect of these sequences probably by enhancing DNA uptake in cells of the reticuloendothelial system [8–10]. A prerequisite to any attempt to inhibit this inflammatory reaction is to identify the organ and the cell responsible for such a secretion *in vivo*.

Nevertheless, the cellular source of TNF-α secretion *in vivo* after intravenous lipoplex injection has not been clearly identified so far. In this work, we studied the biodistribution of the diC14-amidine/DNA lipoplexes [9,11] and we tried to identify the cellular source of TNF-α after lipoplex intravenous injection. Our results suggest the importance of spleen and splenic macrophages in the TNF-α secretion induced by diC14-amidine/DNA lipoplexes and confirm that the preinjection of cationic liposomes modify the physiology of spleen macrophages and their ability to secrete TNF-α after lipoplexes injection.

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doi:10.1016/j.ejpb.2008.01.035

2. Materials and methods

2.1. Mice

Female BALB/C mice used were 6–10 weeks old (Charles River, France). Mice were handled according to institutional guidelines.

2.2. DNA

The pCMV-luc plasmid, containing the luciferase gene under the control of the immediate-early CMV promoter-enhancer, was constructed as described previously [9], purified using a Qiagen Kit (QIAfilterTM Plasmid Giga Kit, Qiagen) according to manufacturer's instructions and resuspended in sterile water. The purified plasmid was further treated with a polymyxin resin (Affi-prepTM, Bio-Rad) to remove endotoxins.

2.3. Liposome preparation

DiC14-amidine (3-tetradecylamino-*N*-tert-butyl-*N*′-tetradecylpropionamidine) was synthesized as previously described [11] and liposomes were prepared as described [9]. Briefly, after dissolving diC14-amidine in chloroform, the solvent was evaporated under a stream of N_2. The resulting lipid film was then hydrated with HBS-20 buffer (20 mM HEPES, 150 mM NaCl, pH 7.3) and the pH was adjusted to 7.3 using HCl. Liposomes were then extruded 7 times through a 0.4 μm polycarbonate filter (Osmonics) at 55 °C. Liposome size is about 200 nm after this step. The same protocol was used for labeled liposomes, except that 1% (molar ratio) of L-3-phosphatidylcholine, 1,2-di[1-14C]palmitoyl ([^{14}C]-DPPC, Amersham Biosciences) was added to chloroform-diluted diC14-amidine before film formation.

2.4. DiC14-amidine/pCMV-luc lipoplexes preparation

All solutions used in the lipoplexes preparation were autoclave-sterilized and warmed at 37 °C immediately before use. The pCMV-luc plasmid DNA was diluted in 0.15 M NaCl to a concentration of 0.5 mg/ml. The diC14-amidine liposomes were diluted in HBS-20 to a concentration of 4 mg/ml in a 4 ml polystyrene tube (Falcon) and protamine sulfate (Sigma–Aldrich) was added to a final concentration of 0.3 mg/ml. A volume of DNA (usually 350 μl) was added into an equal volume of the liposome-protamine mixture while gently shaking the tube. The liposome/DNA mixture was allowed to stand for 15 min at room temperature. The lipoplex was further diluted twice with HBS-10 (10 mM Hepes, 150 mM NaCl, pH 7.3) to generate a lipoplex concentration of 125 μg DNA/ml. Under these conditions, the lipoplex has a cationic lipid/pCMV-luc/protamine ratio of 8:1:0.6 (w:w) and charge ratio was calculated to 4.85 positive charges for 1 negative charge.

Size of lipoplexes was evaluated by photon correlation spectroscopy in a ZetaSizer 1000HS$_A$ (Malvern Instruments, UK), and showed a mean diameter of 500 ± 10 nm.

For *in vitro* experiments, a volume of DNA at 5 μg/ml was added into an equal volume of liposomes (10 μg of diC14-amidine/ml) in DMEM while gently shaking the tube. The liposome/DNA mixture was allowed to stand for 15 min at room temperature before use. These lipoplexes showed a mean diameter of 330 ± 10 nm.

2.5. Biodistribution

Mice were injected via the lateral tail vein with 200 μl of the lipoplex solution (containing 25 μg DNA and 200 μg of labeled diC14-amidine) and, after 10, 30 or 120 min, the mice were bled from retro-orbital sinuses under anesthesia and killed by cervical dislocation. Approximately 150 ml of PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) was injected into the vascular system via the left ventricle in order to wash out residual blood in the organs. Organs were collected and washed by PBS buffer. Tissue extracts were homogenized in water using a tissue-tearor (BioSpec Products). Three hundred microliters of tissue homogenate (or 200 μl for blood) was then added to 2 ml (or 1 ml for blood) of Soluene-350 (Perkin Elmer). Homogenates were then heated at 70 °C for 2 h and, after cooling, 200 μl of hydrogen peroxide (500 μl for blood) was added slowly. After 30 min at 60 °C, 10 ml (15 ml for blood) of scintillating liquid Hionic-Fluor (Perkin Elmer) was added to the samples, then the samples were vortexed and analyzed in a scintillation counter. For control, labeled lipoplexes were added to tissue homogenates or blood of a non-injected mouse, and treated similarly to determine the total radioactivity injected into mice. The data are expressed as a percentage of lipoplexes captured by each organ to the initial dose of lipoplexes injected (= total radioactivity injected). The measurement of the weight of the various organs makes it possible to plot a graph standardized in terms of lipids/g of organs.

2.6. Lipoplexes injection and cytokine assay

Mice were injected via the lateral tail vein with 200 μl of the lipoplex solution (containing 25 μg DNA and 200 μg of diC14-amidine). At indicated times after injection, mice were bled from retro-orbital sinuses under anesthesia and killed by cervical dislocation. Approximately 150 ml of PBS buffer was injected into the vascular system via the left ventricle in order to wash out residual blood in the organs. Organs were collected, washed by PBS buffer and conserved at −80 °C until processing. Tissue extracts were homogenized in PBS buffer containing a cocktail of protease inhibitors (Complete EDTA-free Protease Inhibitor Cocktail Tablets, from Roche) using a tissue-tearor. The cytoplasmic fractions were isolated as the supernatant following centrifugation (10 min at 15 000 rpm, 4 °C) and

stored at −20 °C. The cytokine concentration was quantified in the serum and in organ homogenates using TNF-α ELISA kits (R&D Systems) and normalized to the protein concentration (measured by BCA protein assay, Pierce) for each organ.

2.7. Cell separation by adherence to plastic tissue culture plates

Freshly removed spleens from BALB/C mice were perfused with collagenase at 100 U/ml (Worthington) in Hank's balanced salt solution (HBSS, Invitrogen) then incubated for 30 min in collagenase at 400 U/ml at 37 °C. After incubation, spleens were crushed in HBSS containing 10 mM EDTA at pH 7.4 and cellular suspension was filtered through a cell strainer (Falcon) and centrifuged for 7 min at 1400 rpm. Spleen cells were plated on 24-well plates, in DMEM (Invitrogen) supplemented with 5% FBS (Biowhittaker), 1 mM sodium pyruvate, 1 mM glutamine and antibiotics (Invitrogen). The cell density was 10^7 cells/well for "total cells". For adherent cells, the initial spleen cell input was 10^8 cells/wells in order to reach approximately 10^7 cells/well after removal of non-adherent cells. After 90 min of incubation at 37 °C, non-adherent cells were removed by washing with fresh culture medium and medium was added to adherent cells for a further incubation overnight at 37 °C. After this new incubation, non-adherent cells were removed and plated to new wells. Different cell populations (total cells, adherent cells, overnight (O/N) adherent and non-adherent cells) were then stimulated with diC14-amidine/DNA lipoplexes at a final concentration of 2.5 μg DNA/ml and 5 μg of diC14-amidine/ml in DMEM for 6 h. TNF-α level in the supernatant of cell culture was quantified by ELISA assay (DuoSet, R&D Systems) and normalized to total cellular protein levels (quantified by BCA assay, Pierce).

2.8. Depletion of macrophages

Multilamellar liposomes encapsulating clodronate were prepared as described [12,13]. Briefly, 21.5 mg of egg phosphatidylcholine (Sigma–Aldrich) and 2 mg of cholesterol (Sigma–Aldrich) were dissolved in 10 ml of chloroform and placed in a 100 ml rounded-bottom glass container. The organic solvent was removed under a nitrogen stream in a rotary evaporator and the resulting film was desiccated overnight. Lipid film was hydrated for 10 min under slow rotation in a suspension of 2.5 ml of clodronate solution 0.6 M (Sigma–Aldrich). The solution was allowed to stand 1h30, then sonicated for 3 min at room temperature in a bath sonicator, and incubated for 2 h again. The liposomes were purified by centrifugation (10 min at 15 000 rpm), washed and clodronate-containing liposomes were resuspended in 1 ml of PBS. Mice were injected intravenously with 200 μl of this suspension 24 h before diC14-amidine/DNA lipoplex injection.

Gadolinium chloride (Sigma–Aldrich) was dissolved at 4.5 mg/ml in saline buffer (NaCl 0.15 M) and injected intravenously into mice (0.9 mg per mice) 24 h before lipoplex injection.

2.9. Statistics

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

3. Results

3.1. Biodistribution of lipoplexes in the organs after intravenous injection

We determined the kinetics of distribution of the diC14-amidine/DNA lipoplexes in the blood, lung, liver and spleen after intravenous injection of radioactive labeled diC14-amidine/DNA lipoplexes (Fig. 1A). The lipoplexes were rapidly cleared from the blood-stream since, even in the minutes following injection, only 10% of the injected lipoplexes were found in the blood. The liver and the lung were the main targets: 15% of the amount injected was found in the liver and in the lung 10 min after injection. In spleen, the capture was weaker (5%). A significant reduction of lipoplexes concentration in the lung and blood was observed 2 h after injection, whereas a large fraction accumulated into the liver.

We standardized the kinetics of capture according to the weight of organ tissue. The standardized curve (Fig. 1B), revealed that the spleen was the most efficient organ in capturing lipoplexes 1 h after injection whereas the liver was less efficient.

3.2. Identification of organs involved in TNF-α secretion after intravenous injection of diC14-amidine/DNA lipoplexes

The TNF-α amount was quantified in organ homogenates and in the serum at different time points after diC14-amidine/DNA lipoplex injection (Fig. 2). All organs showed a basal level of TNF-α corresponding most likely to intracellular TNF-α since secreted cytokine had been removed by extensive washing (as described in Section 2). Nevertheless a significant increase in its production could be observed only in the spleen as compared to control mice. TNF-α production did not increase substantially in other organs. In the serum, a significant increase in TNF-α secretion was observed. The kinetics of TNF-α secretion in the serum and the kinetics of intracellular TNF-α production in the spleen were comparable suggesting that TNF-α secreted in serum was originating mainly from the spleen.

3.3. Cells responsible for TNF-α secretion

In order to further identify which spleen cells are capable of secreting TNF-α in response to lipoplex administration, splenocytes were isolated and cell populations were fractionated into adherent cells, overnight non-adherent

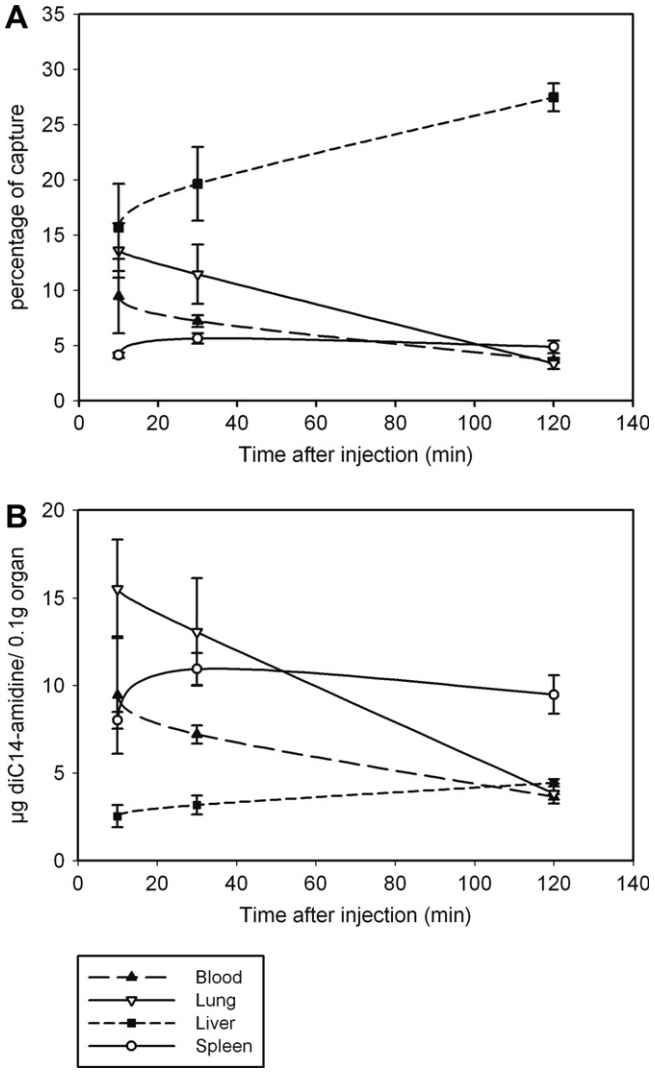

Fig. 1. Biodistribution in organs of diC14-amidine lipoplexes after intravenous injection. (A) Lipoplexes (200 µl) made with diC14-amidine liposomes containing 1% ^{14}DPPC, at a concentration of 125 µg DNA/ml were injected into mice (3 per group). At times indicated, the mice were bled and the organs were recovered to be crushed. The homogenates were then treated and analyzed in a scintillation counter as described in Section 2. (B) Normalized uptake plot as a function of tissue weight (µg/ml for blood (▲)). Data represent means ± SD.

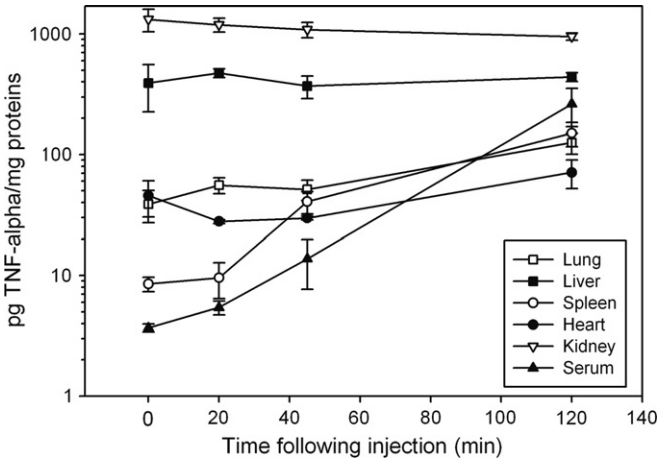

Fig. 2. TNF-α level as a function of time in the organs and the serum after intravenous injection of diC14-amidine/DNA lipoplexes. A complex made of diC14-amidine/DNA/protamine (weight ratio 8:1:0.6), at a final dose of 25 µg DNA/mouse, was injected into mice (3 per group). At different times after injection, Balb/c mice were bled and organs (lung, liver, spleen, heart, kidneys) were recovered. The level of TNF-α was quantified in organ homogenates and in the serum by ELISA assay, normalized to the protein concentration for each organ (expressed as pg/mg protein). TNF-α level in untreated mice was considered as the $t = 0$ time point. Data represent means ± SD. The level of TNF-α in the serum (▲) is expressed as pg/ml serum.

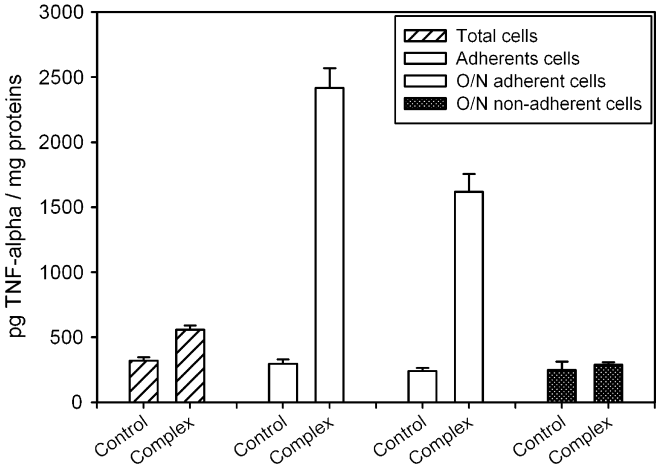

Fig. 3. TNF-α produced in the spleen comes mainly from enriched-macrophage population. Spleen cells were obtained from BALB/C mice, by dissociation of the spleen using collagenase and washed with complete medium. 10^8 cells/well (or or 10^7 cells/well for "total cells") were plated in a 24-well plate (4 wells per group) in complete medium for 90 min. Non-adherent cells were removed and adherent cells were incubated overnight. O/N non-adherent cells were separated from O/N adherent cells and the different populations of cells (total cells, adherent cells, overnight (O/N) adherent and non-adherent cells) were incubated with diC14-amidine/DNA lipoplexes (2:1) with a DNA concentration of 2.5 µg/ml for 6 h. TNF-α level in the cell supernatants was quantified by ELISA and normalized to protein levels. Control = non-stimulated cells. Lipoplex = cells stimulated by diC14-amidine/DNA lipoplexes. Data represent means ± SD.

and overnight adherent cells, taking advantage of differential adherence on plastic culture dishes. Each population of cells was stimulated by incubation with diC14-amidine/DNA lipoplexes and TNF-α secreted in the supernatants was quantified (as described in Section 2). Normalization of TNF-α secretion to total cellular proteins facilitated comparison of the data.

Total splenic cells stimulated with diC14-amidine/DNA lipoplexes secreted small amounts of TNF-α (Fig. 3). After enrichment of adherent cells (mainly dendritic and macrophage cells [14,15]), TNF-α secretion increased significantly as compared to total cells. There was no detectable secretion of TNF-α by cells detached from plastic support after the overnight incubation (mainly dendritic cells [14]) whereas (Fig. 3) an appreciable TNF-α production was observed in overnight adherent cells (macrophages mainly [14–16]).

3.4. Effect of in vivo macrophage depletion on the pro-inflammatory response induced by lipoplexes

We further confirmed the role of macrophages in secretion of TNF-α *in vivo* using macrophage-removing agents: [1] clodronate (dichloromethylene diphosphonate, Cl_2MDP) which is known to deplete macrophages in the spleen and in the liver [12,13] and [2] gadolinium chloride ($GdCl_3$) which acts mainly on liver macrophages since this compound causes in the spleen only red pulp macrophage disappearance and repopulation occurs in the spleen sooner than in the liver [17,18].

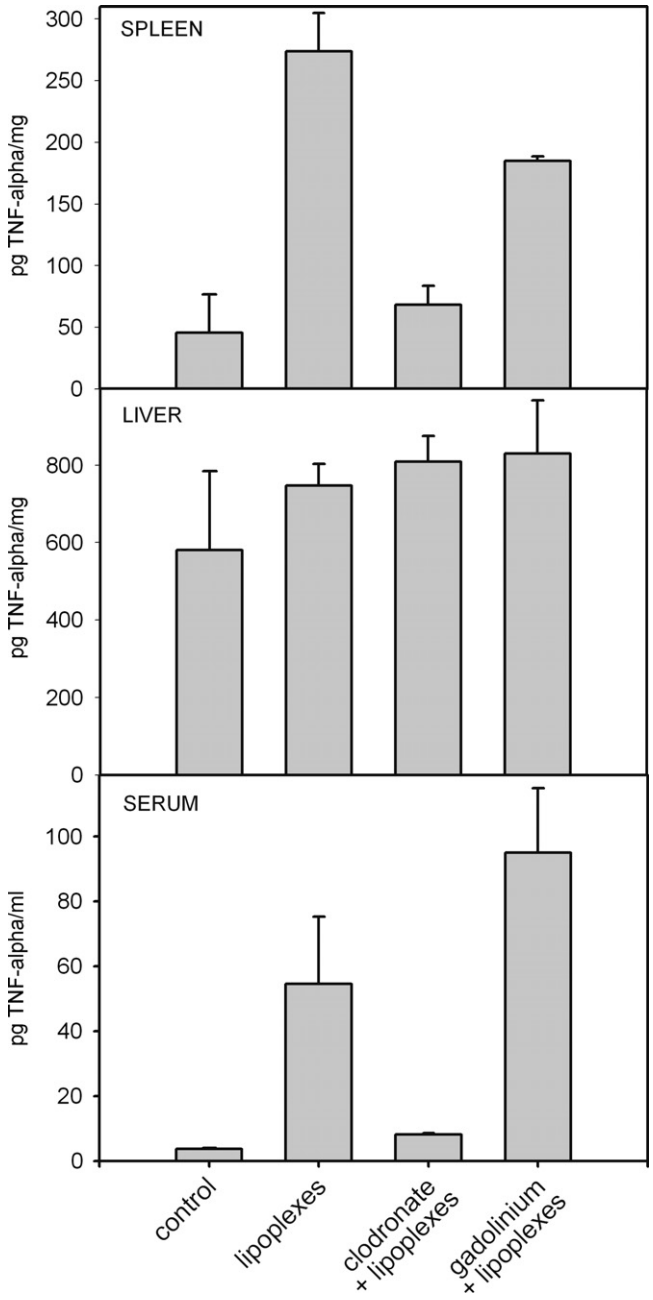

Fig. 4. Role of spleen macrophage in the systemic inflammation induced by diC14-amidine/DNA lipoplexes injection. Mice (3 per group) were injected intravenously with 200 μl of macrophage depleting agent (clodronate 0.6 M or gadolinium chloride 4.5 mg/ml) or with buffer 24 h before diC14-amidine/DNA lipoplex injection prepared as decribed in Section 2. Two hours after lipoplex injection, mice were bled and the liver and the spleen were taken. TNF-α was quantified in the serum and in organ homogenates by ELISA. Control mice did not receive lipoplexes. Data represent means ± SD.

These agents were injected into mice 24 h before injection of diC14-amidine/DNA lipoplexes. Cytokine concentration was measured in the serum and organs homogenates collected from mice two hours after injection of the lipoplexes (corresponding to the maximal secretion of TNF-α into the serum after lipoplex injection). Secretion of TNF-α into the serum was strongly inhibited when mice were pretreated with clodronate but not when gadolinium chloride was used (Fig. 4). The same results were obtained in spleen homogenates, while in the liver, no significant differences were observed between all the treatments. Clearly, depletion of liver macrophages does not modify the TNF-α level either in the liver or in the serum while depletion of spleen macrophages significantly decreases the level of TNF-α in the spleen and serum. This suggests that splenic macrophages are mainly responsible of TNF-α production and secretion into the serum following lipoplex injection.

4. Discussion

DiC14-amidine/DNA lipoplexes injected intravenously induce a strong inflammatory response characterized by secretion into the serum of proinflammatory cytokines like TNF-α [9]. In this report, we showed that the intravenously injected-diC14-amidine/DNA lipoplexes are mainly taken up by spleen and liver cells. The biodistribution profile of lipoplexes was consistent with previous reports obtained with other cationic lipids [5,19–24]. Normalization of the amount of lipoplexes taken up as a function of the organ weight shows that spleen is more efficient than liver at capturing lipoplexes. This could be a consequence of a lower accessibility of liver cells to lipoplexes as compared to spleen cells. A possible explanation for this is that limiting fenestration of the vessels in the liver does not allow an efficient uptake of lipoplexes by Kupffer cells. Another explanation could come from the interaction of liposomes with the serum. Indeed, it has been reported [25] that some serum proteins bound to the liposomes could lead to specific uptake by spleen or liver.

Secreted TNF-α was mainly produced by spleen. This conclusion was supported by several findings. Intravenous injection of diC14-amidine/DNA lipoplexes into mice induced an increase of the intracellular TNF-α in the spleen but not in other organs (Fig. 2). Even though the initial basal level of intracellular TNF-α was higher in other organs such as the liver and the kidneys, the fact that the kinetics of secretion into the serum and the kinetics of intracellular TNF-α production in the spleen were comparable, strongly suggested that the serum TNF-α came mainly from the spleen. The role of splenic macrophages in TNF-α secretion was

supported by the use of macrophage-removing agents. Treatment of mice with clodronate, which is known to deplete splenic and liver macrophages [12,13], resulted in the inhibition of TNF-α secretion into the serum and cell-associated TNF-α production in the spleen (Fig. 4). In contrast, mice receiving gadolinium chloride, which has been reported to deplete mainly liver macrophages [17], had no effect on TNF-α secretion. Why splenic macrophages but not hepatic ones were stimulated by diC14-amidine/DNA lipoplexes remains unclear. It could be due to the low amount of CpG taken up by liver macrophages since the normalized CpG uptake was much lower in the liver (Fig. 1).

We have shown previously [9] that diC14-amidine liposomes, when preinjected intravenously, are able to inhibit the TNF-α secretion induced by diC14-amidine-DNA lipoplexes. This effect could partially be explained by anti-inflammatory properties of diC14-amidine macrophages, demonstrated on the RAW 264.7 macrophage cell line [26]. Indeed, we recently demonstrated that diC14-amidine liposomes inhibit TNF-α secretion induced by both unmethylated CpG DNA sequences and lipopolysaccharides [26]. Surprisingly, free cationic liposomes alone did not to inhibit TNF-α secretion by RAW 264.7 cells after stimulation with unmethylated CpG sequences [26]. Free liposomes acquired their anti-inflammatory activity only when preincubated with serum before incubation with cells. Serum components were separated and lipids of low-density lipoproteins and triglyceride-rich lipoproteins were identified as the serum components that conferred the anti-inflammatory activity to the cationic liposomes [26,27].

As shown in this work, splenic macrophages are most probably the main source of secreted TNF-α induced by intravenous lipoplex injection in mice. Preliminary cytofluorimetric experiments demonstrated that, using the conditions described for lipoplexes, free diC14-amidine liposomes accumulate into spleen macrophages (data not shown). These results confirm that the intravenous injection of diC14-amidine liposomes, targeting mainly tissue macrophages, could inhibit the TNF-α secretion by avoiding the production of TNF-α by these cells and consequently inhibiting secretion of this cytokine in the serum after intravenous lipoplex injection in mice. This means that the use of an anti-inflammatory agent uptaken mainly by spleen macrophages could lead to the reduction of the inflammatory response associated to lipoplexes.

Acknowledgments

Caroline Lonez is a F.R.I.A. fellow (Fonds de la Recherche Scientifique pour l'Industrie et l'Agriculture) and Michel Vandenbranden is Research Associate of the Belgian National Fund for Scientific Research. This work was supported by A.R.C. (Action de Recherche Concertée).

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