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Repeated intraperitoneal injections of liposomes containing phosphatidic acid and cardiolipin reduce amyloid-β levels in APP/PS1 transgenic mice

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Abstract

The accumulation of extracellular amyloid-beta (Aβ) peptide and intracellular neurofibrillary tangles in the brain are two major neuropathological hallmarks of Alzheimer's disease (AD). It is thought that an equilibrium exists between Aβ in the brain and in the peripheral blood and thus, it was hypothesized that shifting this equilibrium towards the blood by enhancing peripheral clearance might reduce Aβ levels in the brain: the 'sink effect'. We tested this hypothesis by intraperitoneally injecting APP/PS1 transgenic mice with small unilamellar vesicles containing either phosphatidic acid or cardiolipin over 3 weeks. This treatment reduced significantly the amount of Aβ in the plasma and the brain levels of Aβ were lighter affected. Nevertheless, this dosing regimen did modulate tau phosphorylation and glycogen synthase kinase 3 activities in the brain, suggesting that the targeting of circulating Aβ may be therapeutically relevant in AD.

From the Clinical Editor: Intraperitoneal injection of small unilamellar vesicles containing phosphatidic acid or cardiolipin significantly reduced the amount of amyloid-beta (Aß) peptide in the plasma in a rodent model. Brain levels of Aß were also affected - although to a lesser extent - suggesting that targeting of circulating Aß may be therapeutically relevant of Alzheimer's disease.

Key words: Amyloid-β; Alzheimer treatment; Nanoparticles; APP/PS1

Alzheimer's disease (AD) is the most common neurodegenerative disorder, and it is characterized by two key pathological modifications in the brain: the deposition of extracellular amyloid plaques and the formation of intracellular neurofibrillar tangles.[1] Amyloid plaques mainly contain the amyloid-beta (Aβ) peptide that is released by the proteolytic cleavage of the amyloid precursor protein (APP).[2] Aβ, primarily containing 40 or 42 amino acid residues ($A\beta_{1-40}$ and $A\beta_{1-42}$, respectively), can form monomers, oligomers, fibrils and plaques.[3] However, the oligomers are believed to be the most toxic form of the Aβ peptide.[4]

Most cases of AD pathology are *idiopathic* (of unknown cause), although a small proportion of cases are genetic and known as the Familial Alzheimer Disease (FAD). FAD may be either late or early onset, and it is generally caused by mutations in the APP,[5] Presenilin 1[6] or Presenilin 2[7] genes. Transgenic mouse models expressing one or two of these human mutations have been developed and used as models of FAD. In these transgenic mouse models, the expression of human APP with a single or double mutations, leads to the accumulation of Aβ and

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the formation of plaques, resembling the amyloid pathology evident in the brain of AD patients. Thus, these transgenic AD models would appear to be useful tools to evaluate the potential of therapeutic agents that target Aβ. In fact, different therapeutic approaches have been assessed in these models of AD and in some cases; reductions of the final amyloid burden have been reported.[8-10]

In its protective role, the blood brain barrier (BBB) complicates the delivery of many potentially important therapeutic drugs. To reduce Aβ levels in the brain we can avoid the BBB because it was hypothesized that a dynamic equilibrium exists between the brain and peripheral pools of Aβ.[8] Brain Aβ clearance may be accomplished through transporter proteins[11] and accordingly, the equilibrium between brain and peripheral Aβ has been adopted as the basis for several therapeutic interventions. For instance, sequestering or modifying free peripheral Aβ would shift the equilibrium towards enhanced efflux, potentially producing a reduction in brain Aβ and the accumulation of plaques, an approach that has been referred as the 'sink-effect'.[12,13]

Increasing attention has been given to the therapeutic use of nanoparticles, particularly as vehicles for the delivery and targeting of drugs.[14] Among these, the versatility of liposomes (LIPs) has drawn considerable attention to their potential uses, for a very long time in cancer, and more recently in neurological diseases (for review,[15]). Indeed, we recently demonstrated that LIPs with phosphatidic acid (PA-LIP) or cardiolipin (CL-LIP) incorporated into their bilayer could bind Aβ with high affinity *in vitro*.[16] Hence, we have used these vesicles to explore whether the 'sink-effect' can be induced in young APP/PS1 transgenic mice following repeated intraperitoneal injection. Our results show that such treatment significantly reduces the amount of Aβ in the plasma of mice, while achieving a minor reduction in brain Aβ. However, these treatments did significantly alter the tau phosphorylation and GSK3 activity in the brain.

Methods

Experimental animals

The double transgenic mice used in the present study incorporate a human APP construct bearing the Swedish double mutation and the exon-9-deleted PSEN1 mutation: B6.Cg-Tg (APPSwe, PSEN1dE9) 85Dbo/J (Jackson Laboratory, Bar Harbor: stock no. 005864). The genotype of the mice was confirmed by PCR of DNA isolated from tail biopsies[17] and we used only 3 month old male mice to reduce the variability in the results. All animal care and handling strictly followed the current Spanish legislation and guidelines, and those of the European Commission (directive 2010/63/EU).

Liposome preparation and characterization

Liposomes were composed of Sphingomyelin and Cholesterol (Sm/Chol, 1:1 molar ratio), containing either 5 mol% of PA or CL. Small unilamellar vesicles were prepared by repeated extrusion of the initial multilamellar preparations through polycarbonate filters (100-nm pore size diameter, Millipore Corp., Bedford, MA), as described previously.[16] The integrity of the liposomes was studied by measuring the retention of liposome-entrapped fluorescent dye calcein, as described previously.[18] Size, polydispedity index and Z-potential were determined using a ZetaPlus particle sizer and ζ-potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, U.S.A.).

Liposome binding to Aβ peptides

The $A\beta_{1-40}$ peptide (Phoenix, Inc, USA) was reconstituted according to the manufacturer's instruction. To obtain a preparation enriched in Aβ oligomers, the monomeric peptide solution was diluted with phosphate buffered saline (PBS) 1 × to 100 μM and left for 48 h at 37 °C. After incubation, large aggregates of Aβ were removed by ultracentrifugation (179,000 × *g*: Beckman Optima Max, Beckman MLS 50 rotor) for 30 min at room temperature (RT), and the presence of small soluble aggregates was assessed by AFM, as described.[19] The binding of the Aβ peptides to the liposomes was studied by using liposomes radiolabeled with [^{3}H]-Sm (0.001 mol% of total lipids) and through ultracentrifugation on a discontinuous sucrose density gradient, as described elsewhere.[16] The distribution of lipids along the gradient was followed by counting the liposome-associated radioactivity by liquid scintillation. The distribution of the peptide was followed by an established dot-blot procedure[16] using mouse monoclonal antibody 6E10 (1:1000, Signet Dedham, MA). For the $A\beta_{1-40}$ detection, chemiluminescent spots were semi-quantitatively estimated using ImageQuant LAS4000 and the proportion of the bound peptide was expressed as the ratio between the amount of peptide in fractions 1-5 over the total amount of peptide.[16]

The effect of liposomes on cell proliferation

The possible effect of liposomes on cell proliferation was evaluated in cultured hCMEC/D3 and human neuroblastoma SH-SY5Y cells. The hCMEC/D3 cells recovered between passage 25 and 35 were seeded at a density of 27,000 cells/cm^2 in tissue culture flasks coated with 0.1 mg/mL rat tail collagen type 1 and they were cultured at 37 °C in an atmosphere of 5% CO_2/saturated humidity in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with: 5% v/v fetal bovine serum (FBS), 1 U/ml Penicillin, 100 μg/ml Streptomycin, 1.4 μM hydrocortisone, 5 μg/mL ascorbic acid, 1/100 chemically defined lipid concentrate (Invitrogen), 10 mM HEPES and 1 ng/mL basic FGF (bFGF).[20] SH-SY5Y cells were cultured in a 5% CO_2 humidified incubator at 37 °C in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). The cells were differentiated over 7 days with 10 μM retinoic acid (RA) dissolved in dimethylsulfoxide (DMSO) and diluted in culture medium to reach DMSO concentrations previously demonstrated to be non-toxic.[21]

Cells were exposed to liposomes (6 to 120 μg lipid/mL) for 24 h at 80%-90% confluence and untreated cells were used as a negative control. After incubation with liposomes, proliferation was evaluated by the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay,[22] and each sample was analyzed in triplicate.

Animal treatment

Transgenic male mice (3 months old, n = 7 per group) were injected intraperitoneally with 50 μl (2.5 mmol) of each liposome preparation every other day for three weeks. There were four treatment groups: phosphatidic acid-liposome (PA-LIP), cardiolipin-liposome (CL-LIP), Plain-liposome (LIP) and PBS as controls. Two wild-type mice were included in each group to evaluate possible toxic effects and no mice displayed adverse effects in any of the assays. Two independent experiments were performed and the results are presented as the mean of all mice analyzed.

Blood and tissue sampling

Blood samples were taken before (t0) and after the treatment (at the end-point, 24 h after the last injection point) by submandibular vein puncture.[23] After mixing with 10 mM EDTA in PBS 1:1, the blood was centrifuged for 5 min at 10,000 × *g* at RT to obtain plasma,[24] which was stored at −80 °C.

Mice were sacrificed in CO_2 and a necropsy was performed by personnel expert in handling laboratory animals. The brains were removed and split into two hemibrains, as described previously.[25] Briefly, one hemibrain was snap frozen on dry ice for subsequent homogenization and stored at −80 °C. The other hemibrain was fixed for 24 h in 4% paraformaldehyde (PFA) in PBS and cryoprotected in graded concentrations of sucrose (15%-30%) in PBS. The fixed tissue was then set in O.C.T.™ compound (Tissue-Tek® Sakura) and frozen at −80 °C.

Aβ quantification by ELISA (1-40 and 1-42)

Brain tissue was homogenized in 8 vol of ice-cold guanidine buffer (5 M guanidine HCl/50 mM Tris HCl, pH 8). The homogenates were mixed for 3 h at room temperature (RT) and stored at −20 °C. Brain homogenates were diluted 1:50 in albumin-PBS-Tween buffer (5% v/v Bovine Serum Albumin, 0.03% w/v Tween-20 in PBS) prior to centrifugation at 16,000 × *g* for 20 min at 4 °C. The amyloid levels were measured in plasma samples and diluted brain supernatants with an Aβ40 or Aβ42 Human ELISA kit (Invitrogen, USA) according to the manufacturer's instructions. The absorbance in the plates was read at 450 nm on an Opsys MR microplate reader (Dynex Technologies, USA).

Complement assay

The details of the serum preparation, characterization and functional assessment of complement pathways were as indicated in our previous studies.[26] Due to the substantial biological variation of complement proteins in serum, and the large number of positive and negative feedback interactions, we separately assessed the generation of complement activation products in the sera from five healthy individuals.[27]

Gel electrophoresis, Western blotting and immunodetection

Brain tissue was homogenized in 3 vol of ice-cold lysis buffer (20 mM Hepes, 100 mM NaCl, 100 mM NaF, 1 mM $NaVO_4$, 5 mM EDTA, 1% Triton X100) with proteases inhibitor cocktail (Roche Diagnostic) and 1 μM okadaic acid (Calbiochem) as phosphates inhibitor. The homogenate was then centrifuged at 4 °C for 20 min at 16,000 × *g* and the supernatant was isolated. The protein concentration was measured using the BioRad DC Protein Assay (BioRad) following the manufacturer's protocol and prior to resolving the proteins, loading buffer (10% SDS, 0.5 mM DTT, 325 mM TrisHCl [pH 6.8], 87% glycerol, bromophenol blue) was added to the supernatants. Protein extracts of the brain homogenates were resolved on sodium dodecyl sulphate polyacrylamide gels (SDS/PAGE: 20 μg protein/lane), and transferred to a nitrocellulose membrane (Whatman, Germany). The membrane was incubated in a 10% (w/v) solution of non-fat milk for 1 h at RT. After overnight incubation at 4 °C with the primary antibody (more detail in supplementary section), the blots were washed in 0.1% w/v Tween-PBS and incubated with the horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, USA), which was detected by Western Lightning™ Chemiluminescence (Perkin Elmer, USA). β-Actin served as the internal control and the intensity of each band was determined with ImageJ™ software (NIH, USA).

Immunofluorescence studies

Freezing cryostat coronal sections (30 μm) of fixed hemibrains were obtained around Bregma-2 (Leica, Germany). To perform immunofluorescence assays, pretreated slices with formic acid 70% were incubated with the primary antibodies, 6e10 (1:1000, COVANCE) and GFAP (1:500, Promega) overnight at 4 °C. After that, the sections were washed and incubated with anti-rabbit Alexa 488 and anti-mouse Alexa 555 conjugated secondary antibodies (Invitrogen). After washing, DAPI was used to label the cell nuclei and the sections were mounted with Fluoromount G (Southern Biotech). Finally, sections were visualized by fluorescence microscopy (Axiovert200, Zeiss) and images were captured using a SPOT RT Slider camera (Diagnostic). Analyses were performed using ImageJ™ software.

Statistical analyses

Sigma Plot software was used for all statistical analyses. Student's t-test was used to compare results in paired groups, treated (PA-LIP or CL-LIP) and control (LIP + PBS). In all cases, differences were considered statistically significant when $P \leq 0.05$ (*), $P \leq 0.01$ (**) or $P \leq 0.001$ (***).

Results

Characterization of liposomes

Based on three independent measurements, the mean liposomes size was 102 ± 2 nm and they remained stable for 72 h. The polydispersity index of the liposomes was 0.01 and when assessed by DLS, the calculated ζ-potential values were −25.06 ± 1.5 mV for plain-liposomes and −28.55 ± 1.2 mV for liposomes carrying PA or CL. Calcein-entrapped liposomes were stable and retained their content over 24 h incubation in cell culture medium or human plasma (data not shown).

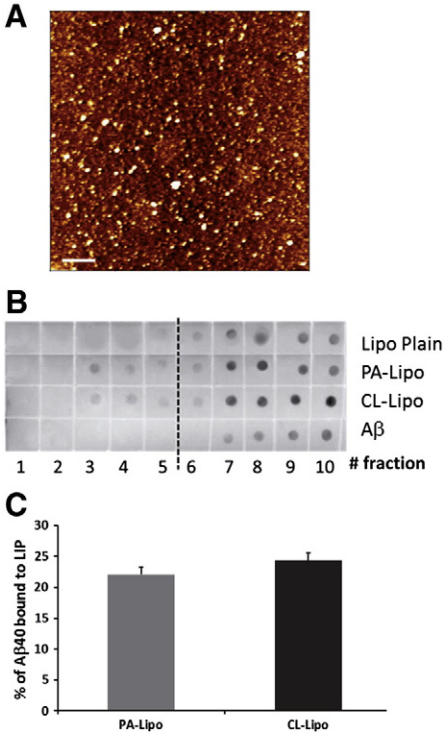

Figure 1. Binding of liposomes to Aβ peptide by ultracentrifugation assay. **(A)** AFM analysis of $A\beta_{1\text{-}40}$ aggregates. A representative 4 × 4-μm x-y, 2-nm total z-range AFM image is shown. Bar: 500 nm. **(B)** The peptide distribution along the gradient fractions was followed by a dot-blot procedure and a representative dot-blot is shown. **(C)** The proportion of peptide bound to liposomes was expressed as indicated in the text. Results show the means ± s.e.m. (n = 3).

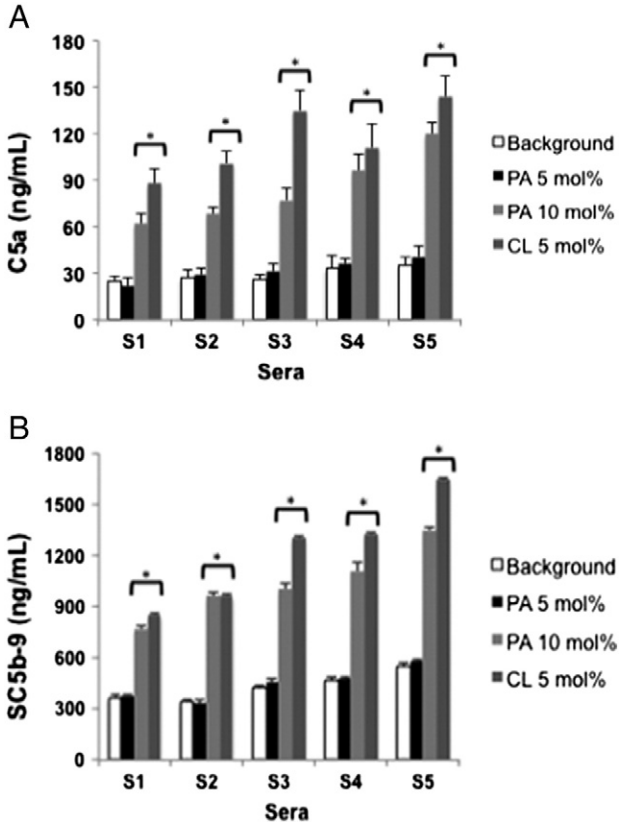

Figure 2. Liposome-mediated complement activation in human serum.Panels **(A)** and **(B)** compare the liposome-mediated rise of serum anaphylatoxin C5a and the soluble complement activation product SC5b-9 in the serum from five healthy individuals (S1-S5), respectively. In all tests, the liposome concentration was 3.5 mg total lipid/mL serum, and in all the sera, zymosan (1 mg/mL) treatment generated more than 35 μg/mL and 1200 ng/mL SC5b-9 and C5a, respectively: $*P \leq 0.05$ compared with background and liposomes containing 5 mol% PA in their bilayers (saline-treated serum).

Binding of liposomes to Aβ peptide

The binding of PA and CL functionalized liposomes to $A\beta_{1\text{-}42}$ has been established previously.[16] Liposome binding to $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$ (as positive control) was assessed by ultracentrifugation on a discontinuous sucrose density gradient, measuring the amount of peptide bound to the liposomes. PA or CL liposomes enriched vesicles bound approximately 25% of either of the peptides tested (Figure 1).

In vitro evaluation of the effect of liposomes on cell viability

The effects of different doses of liposomes on hCMEC/D3 and SH-SY5Y cell proliferation were determined using the MTT assay. None of the liposomes tested altered the proliferation of either cell type at the lipid concentrations tested (data not shown).

Activation of the complement system

We tested whether Aβ binding liposomes can trigger complement activation in human sera. Liposomes containing either 10 mol% PA or 5 mol% CL activated complement in all the sera tested, as evident through the rapid rise in both C5a anaphylatoxin and SC5b-9 (the vitronectin-bound form of the membrane attack complex C5b-9) above background levels (Figure 2). However, lowering the proportion of PA to 5 mol% neither compromised Aβ-binding[16] nor induced complement activation (Figure 2). Indeed, even when human serum was spiked with $A\beta_{1\text{-}40}$ (5 μg/mL serum), the addition of Aβ-binding PA (5 mol%) liposomes did not trigger complement activation (data not shown).

Effect of liposome administration on plasma and brain Aβ levels

To determine the basal level of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, blood and plasma were obtained before commencing the injection regime (**t0**), and the putative effect of the treatment was ascertained in samples taken at the end-point (**tf**), 24 h after the last injection. The results, represented as tf-t0 (Figure 3, *A-B*), highlighted a significant reduction in both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in plasma after PA-LIP and CL-LIP administration. PA-LIP provoked a 6-fold reduction in $A\beta_{1\text{-}40}$ ($P < 0.05$) and a 3-fold decrease in $A\beta_{1\text{-}42}$ ($P < 0.05$). Higher reductions in $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, 10- and 7-fold respectively, were evident in CL-LIP treated mice ($P \leq 0.001$ and $P < 0.05$).

The effects of both types of liposomes on brain amyloid burden were also tested using the same ELISA kit. The results showed decreasing levels in both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ burden in animals that

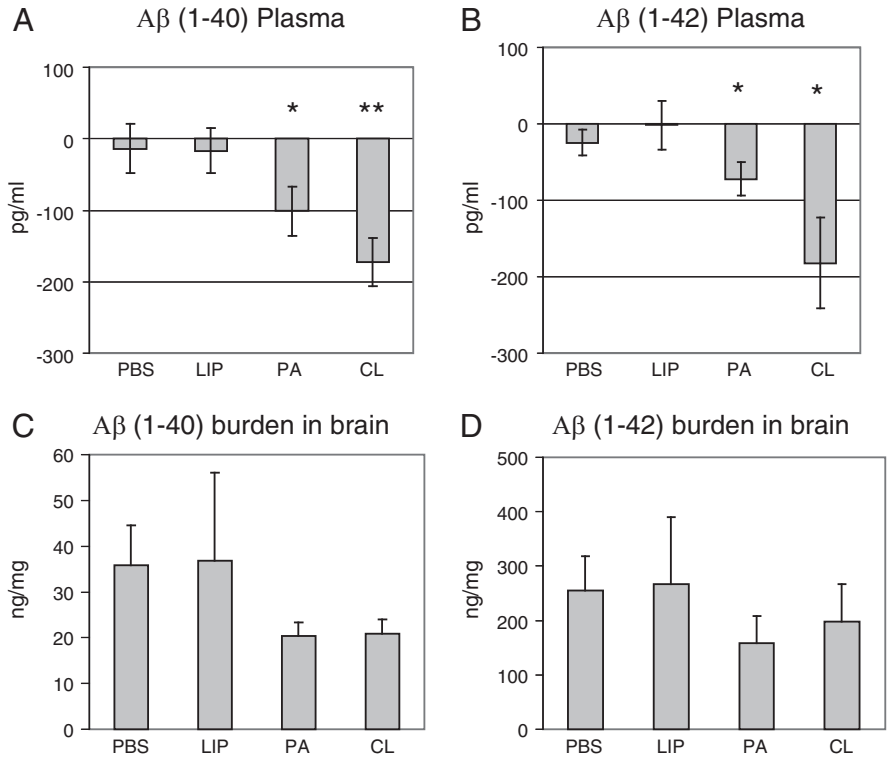

Figure 3. Amyloid-β in the plasma and brain of the treated transgenic mice. **(A-B)** The reduction of human $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in plasma is shown as the difference between post- and pre-treatment levels (tf-t0) in pg/ml of plasma. **(C-D)** Human $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ burden in the brain evident through the direct determination of the final amount of peptide in the brain of the treated mice in ng/mg of brain mass. The graph represents the mean ± s.e.m.

received PA-LIP ($p = 0.055$ and $p = 0.07$, respectively). CL-LIP treated mice showed similar results in $A\beta_{1\text{-}40}$ levels ($p = 0.06$), however $A\beta_{1\text{-}42}$ was not statistically significant (Figure 3, *C-D*).

The number, size and distribution of plaques are important parameters in AD that can be altered by therapeutic intervention. To confirm the ELISA results and to better understand the effects of the liposomes, immunofluorescence analysis was performed on fixed brain tissue (Figure 4). The presence of amyloid deposits (red spots indicated by white arrowheads) appeared to be more prominent in the control animals, however these differences were not statistically significant (Figure 4). Similarly, the number of GFAP-expressing cells (green) at this age was not sufficiently high to observe qualitative differences between treatments. These data were corroborated in Western blots.

Differences in Aβ production

We determined whether the holoAPP, its proteolytic products or other elements relevant in the generation of Aβ, such as BACE, were modified by the administration of the liposomes. No major differences in total APP levels (22C11) or of its products (6E10) were evident in western blots among mice treated with LIP, CL-LIP or PA-LIP (Figure 5, *A*). Similar levels of BACE were found in all groups, suggesting that the activity of this enzyme and APP proteolysis were not affected. These data confirm that amyloid peptide production was not modified by changes in either holoAPP protein or that of BACE.

Synaptic alterations and glial reaction

Any treatment for Alzheimer's disease should improve synaptic activity and thus, we tested the effect of liposome treatment on some synaptic markers like PSD95, P-synapsin and p120. No major differences in PSD95 were evident in western blots (Figure 5, *B*), although the more p120 was evident in the mice treated with CL-LIP ($P \leq 0.01$) or PA-LIP (not statistically significant).

In the transgenic mouse strain studied here a glial reaction around the plaques is normally detected and this reaction augments as the density of plaques increases.[25] However, we found no modification in the glial reaction by immunofluorescence or in western blots comparing treated and control groups (Figures 4 and 5, *B*).

Effect of the liposomes on Tau phosphorylation

We finally evaluated the tau protein and its phosphorylation using specific antibodies. This post-translational modification is regulated by a plethora of kinases[28,29] and some phosphatases.[30] We found that the degree of tau phosphorylation was modified by treatment with either PA-LIP or CL-LIP and we detected a statistically significant reduction in the level of AT100 and AT270 (Figure 6, *A*) after treatment with either PA-LIP ($P < 0.01$) or CL-LIP ($P < 0.001$). By contrast, there were no major differences in the internal controls (total tau-pan-tau- and β-actin). We tried to correlate the differences observed with altered kinase and phosphatase activity relevant to tau[29,30] but no

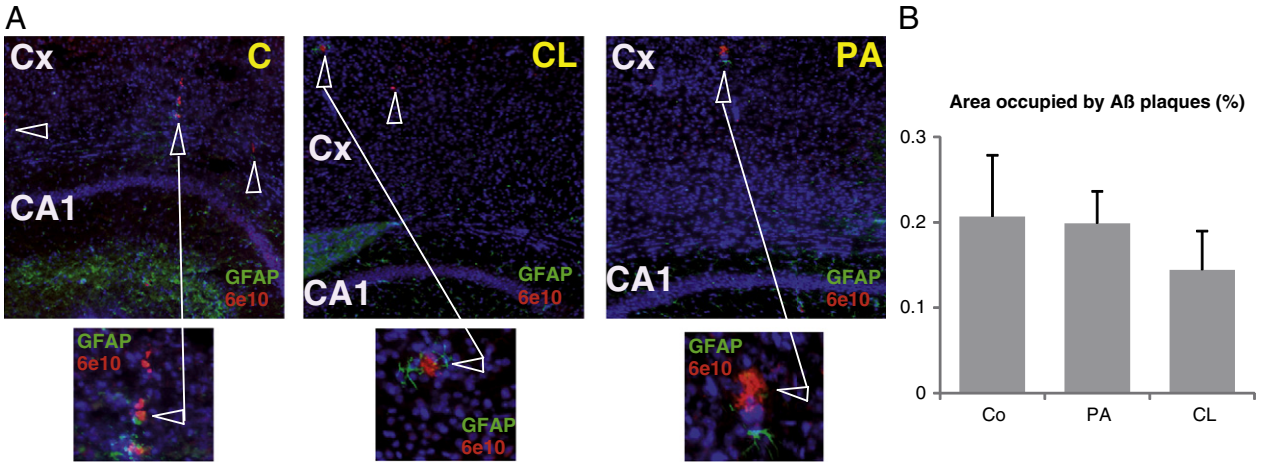

Figure 4. Immunofluorescence analyses of the treated and control mice brain. **(A)** Coronal fixed section from a brain treated with liposomes containing **PA**, **CL** or with plain-liposomes (**control**) and stained with an antibody against APP (6E10, red) and GFAP (green). In addition, the sections were counter-stained with DAPI (blue). In the section, arrowheads indicate the accumulation of amyloid (red spots) in regions of the cortex (**Cx**) or hippocampus (**CA1**). **(B)** Percentage of the brain area occupied by plaques showed not statistically significant differences between groups.

substantial differences were evident (Figure 6, *B*). Finally, we determined the amounts and activation status of GSK3, SAP/JNK and p38MAPK, using specific antibodies and phospho-antibodies. Our data showed that pGSK3Tyr 216/276 and pSAP/JNKTyr138/185 were significantly diminished (Figure 6, *C*) by exposure to PA-LIP ($P < 0.01$) and CL-LIP ($P < 0.05$).

Discussion

In this study we have assessed the benefits that may be gained by administering liposomes that can bind to Aβ and initiate the "sink effect" in a mouse model of AD. While intraperitoneal injection of a small fraction of liposomes will be cleared by resident macrophages in the peritoneal cavity, at higher concentrations a significant fraction of vesicles can drain through the stomata in the diaphragm to reach the lymphatic system, and eventually the bloodstream.[31] From the stomata, the peritoneal fluid enters the subperitoneal lymphatic lacunae. The principal route of extrinsic lymphatic drainage is *via* parasternal lymphatic trunks that carry lymph and suspended vesicles to the parasternal/mediastinal lymph nodes and ultimately, to the right lymphatic or upper terminal thoracic duct. By contrast, liposome extraction by macrophages in the regional lymph nodes remains marginal.[32,33] The anionic liposomes we used are well known to be cleared by macrophages of the reticuloendothelial system.[34] Here, possible accelerated clearance could even enhance Aβ translocation to macrophages. For these reasons, we introduced liposome intraperitoneally (slow appearance in the blood) to allow gradual interaction with Aβ. Accordingly, peritoneal injection may represent a convenient way to repeatedly administer liposomes and their introduction into the bloodstream.

Our results *in vivo* clearly show that both liposome treatments can reduce Aβ levels in the circulating blood pool. This further confirms the ability of these liposomes to interact sufficiently with Aβ prior to their rapid clearance by hepatic and splenic macrophages. Our hypothesis is that liposomes act by removing the peripheral Aβ peptides (in the blood circulation) drawing out the central soluble Aβ aggregates.

Indeed, anionic liposomes are prone to complement opsonization[31] and clearance by macrophages in contact with blood.[35] However, we were able to overcome complement activation by PA containing liposomes by reducing the PA content to 5 mol% without compromising their Aβ-binding properties. Indeed, these liposomes still failed to trigger complement activation even after Aβ binding. This is also advantageous, since inadvertent activation of the complement system can elicit acute and pro-inflammatory responses. Indeed, such reactions have been noted following infusion of many nanomedicines to animals and humans.[35,36]

It has been suggested that soluble Aβ can cause abnormal vascular reactivity in the absence of vascular deposition or vessel wall dysfunction.[37] Therefore, it is tantalizing to propose that liposome-mediated reduction of Aβ in the blood could represent a suitable therapeutic strategy to prevent frequent strokes due to the amyloid deposition in small arteries and capillaries in vessels affected by amyloidosis.[38]

In these experiments we have used the APP/PS1 transgenic mouse line, one of the most extensively studied mouse models of AD.[39] This line has been amplified and characterized over the past 4 years and during this period, we have detected the presence of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in blood and brain, as well as the presence of *amyloid plaques* and their increase as adult mice mature (data not shown). Thus, we were aware that mild amyloid deposits appear in the brain of mice in our colony at 3 months-of-age. Plaques augment exponentially for some months (number, area and density), reaching a plateau at around 9-12 months with a slower increase thereafter (data not shown). This information suggests that 3 month old mice represent a good age to begin to treat this transgenic colony and to study the effect of our formulation on early development of amyloid plaques.

Following liposome administration, we noticed minor reductions in the Aβ burden in the brain of the mice, although these were modest. The amyloid levels in the brain were only measured after the treatment regime had terminated and

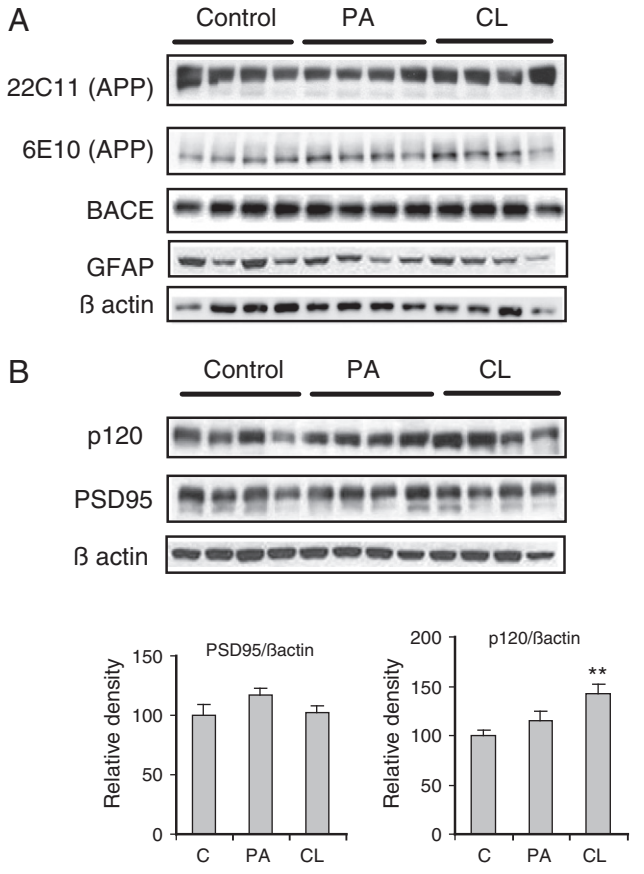

Figure 5. Western blot analysis of Aβ production and markers of synapses. Four samples from each group were separated by electrophoresis and analyzed in western blots. **(A)** Images showed no major differences in the full length APP (22C11 and 6E10 antibody). Similarly, no noticeable differences were detected in BACE or GFAP. **(B)** Analysis of some synaptic proteins showed that PSD95 was not modified but there was an increase in p120 after CL-LIP treatments. β-Actin was used as a loading control. The graph represents the normalized values of the densitometric analysis (being the control 100%) represented as the mean ± s.e.m (** $P \leq 0.01$).

accordingly, we could not compare the true effect of liposome treatment on brain Aβ levels and hence, the sink effect. It should be emphasized that this transgenic mouse model displays important variations in brain amyloid levels, even among *littermates* and independently of age. Therefore, we cannot rule out that longer treatment could produce better results and a more important reduction in Aβ brain levels. Moreover, it is thought that circulating Aβ peptides act as prion-like proteins, inducing deposition,[40] and therefore, any reduction in these peptides will provoke a decrease in plaque accumulation.

Further, we have examined whether the differences in both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in plasma were associated with some key biochemical differences in the brain, and in neurons. We found no major differences in the levels of proteins responsible of Aβ production, such as APP or BACE, indicating that the reduction of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels is not due to differences in the substrate or in the level of its processing. This strongly indicates that the reduction is due to the clearance of amyloid rather than its production, in part because BACE activity has been associated with the amount of protein[41] and γ-secretase production is strongly enhanced by the mutated human transgene (PS1).

These APP/PS1 transgenic mice have been reported to show disturbances in synapsis independent of the accumulation of amyloid, possibly due to the overexpression of APP.[42] However this alteration may recover after different treatments[43] and we tested whether liposome administration could reverse such destabilization of synapses. Accordingly, we have analyzed one important axonal protein, PSD95, which enhanced the maturation of synapses,[44] and the presynaptic p120 that fulfils an important role in the stability of cell-cell adhesions.[45] We failed to detect a clear variation in PSD95 levels, despite the fact that some authors have detected an increase in this protein in APP/PS1 mice that may compensate for the deficient synaptic activity.[46] On the another hand, p120 is thought to play a central role in the γ-secretase association and processing of cadherins, and it may suppress APP processing and Aβ production, possibly by recruiting γ-secretase to cadherins and thereby, limiting its availability for the processing of APP.[47] The accumulation of this protein detected in our treated mice, even being slight, could collaborate in the reduction of Aβ levels in the brain.

Finally, tau, some phospho-tau epitopes, and some kinases and phosphatases relevant for tau dynamics were analyzed, given that tau phosphorylation and its subsequent accumulation as *tangles* are the other important hallmarks of AD.[48,49] Again our data showed that some phospho-epitopes of tau, such as PHF-1, may diminish, yet tau phosphorylation at AT100 and AT270 was more clearly modified by both liposomes. The differences in these phospho-epitopes are apparently not due to differences in PP2A as the activation status of PP2A appears not to be modified by the treatments. Moreover, the analysis of some kinases relevant to tau metabolism, such as GSK3 and SAP/JNK, showed a reduction in Tyr- and Thr/Tyr-phosphorylation after treatment. In both cases, this phosphorylation correlates with kinase activation, which was reduced in correlation with the tau-1 decrease.[49,50] It is interesting to note that the CL-LIP and PA-LIP did not produce similar responses and while PA-LIP treatment generated weaker p-tau modification, there was a clear reduction in pGSK3-pTyr and SAP/JKN-pThr/Tyr. By contrast, the reduction in AT100 and AT270 was more important following CL-LIP administration, although the modification of the kinases analyzed was milder. All these biochemical data suggest that each liposome composition has a distinct impact on the final effect, which opens many interesting questions about the secondary effects on other tissues/metabolites that may generate slightly different brain responses that must be taken into account.

Together, the data presented here indicate that liposomes containing PA or CL can alter circulating amyloid and in addition, directly or indirectly, they can modify brain metabolism. Obviously, whether the reduction in brain amyloid observed is at least partially due to this metabolic modification remains unclear. In summary, our data strongly suggest that liposomes containing PA or CL may serve as interesting therapeutic agents to reduce Aβ in the peripheral blood and subsequently, this peripheral reduction in Aβ may modify the final Aβ levels in the brain.

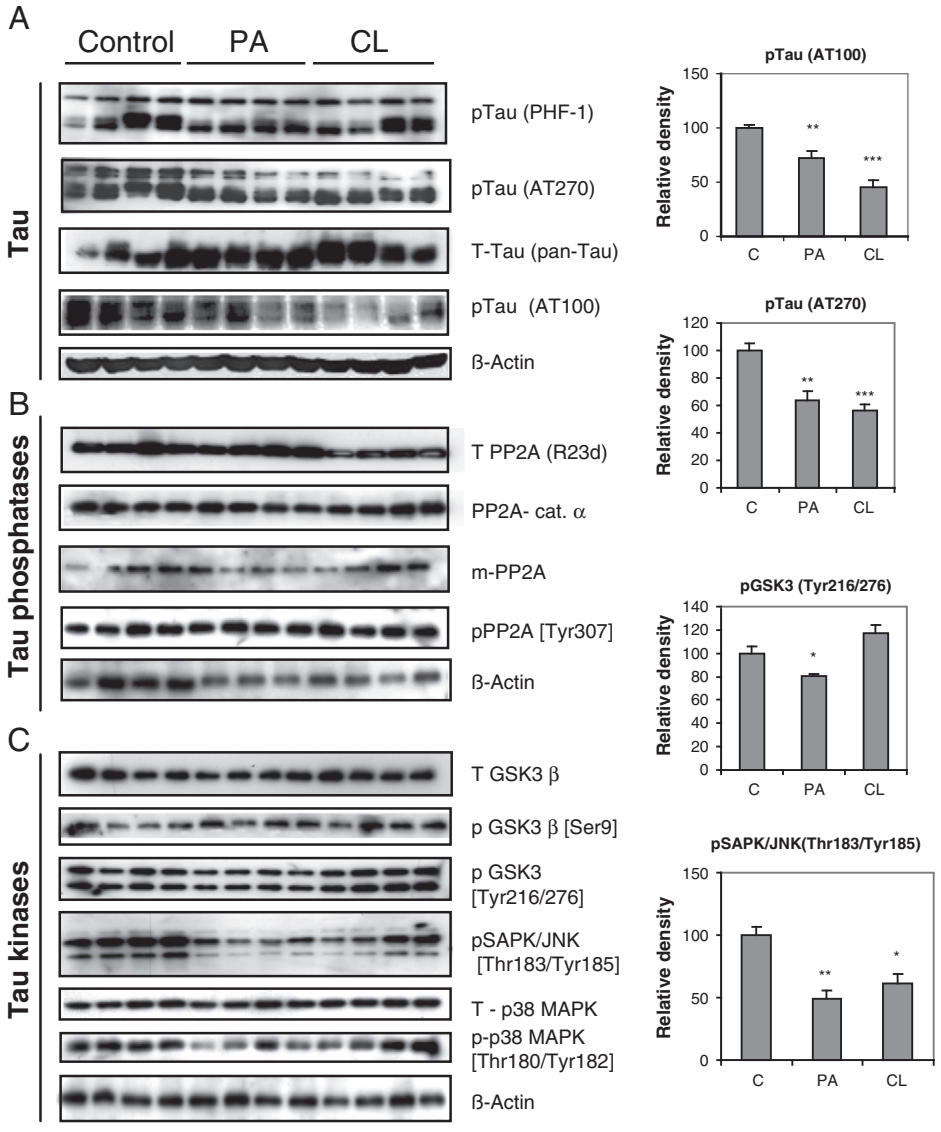

Figure 6. Tau, kinases and phosphatases levels in brain extracts.Some representative blots from three repeated experiments normalized with actin (previously controlled loading with Ponceau staining). **(A)** Westerns blot of total tau (pan-tau antibody) and some phosphoepitopes of tau (antibodies PHF-1, AT270 and AT100) using β-actin as a loading control. Major differences were detected in AT100 and AT270 after CL-LIP treatment. The graph on the right represents the normalized values of AT100 represented as the mean ± s.e.m (** $P \leq 0.01$, *** $P \leq 0.001$). **(B)** In western blot analysis of PP2A (total level, methylated or phosphorylated level of catalytic subunit), no consistent differences were detected during the treatment or in the different groups of mice. β-Actin was used as loading control. **(C)** In western blots of some tau kinases and phospho-epitopes of the active or inhibited form of these kinases (Total GSK3, pGSK3 $^{Tyr216/276}$ and pGSK3^{Ser9}; total p38MAPK and the active p38MAPK $^{Thr180/Tyr182}$; and the active SAP/JNK $^{Thr183/Tyr185}$), the activation of GSK3 and JNK was modified by PA-LIP administration. The graphs on the right represent the normalized values of pGSK3$^{Tyr\ 216/276}$ and pSAP/JNK $^{Thr183/Tyr185}$ presented as the means ± s.d. (* $P \leq 0.05$, ** $P \leq 0.01$). β-Actin was used as a loading control.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.09.015.

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