

Liposome-based immunotherapy against autoimmune diseases: therapeutic effect on multiple sclerosis

Aim: Based on the ability of apoptosis to induce immunological tolerance, liposomes were generated mimicking apoptotic cells, and they arrest autoimmunity in Type 1 diabetes. Our aim was to validate the immunotherapy in other autoimmune disease: multiple sclerosis. **Materials & methods:** Phosphatidylserine-rich liposomes were loaded with disease-specific autoantigen. Therapeutic capability of liposomes was assessed *in vitro* and *in vivo*. **Results:** Liposomes induced a tolerogenic phenotype in dendritic cells, and arrested autoimmunity, thus decreasing the incidence, delaying the onset and reducing the severity of experimental disease, correlating with an increase in a probably regulatory CD25⁺ FoxP3⁺ CD4⁺ T-cell subset. **Conclusion:** This is the first work that confirms phosphatidylserine-liposomes as a powerful tool to arrest multiple sclerosis, demonstrating its relevance for clinical application.

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Liposomes are small vesicles with an aqueous core encapsulated by a lipid bilayer that constitute a promising drug delivery system [1]. Liposome formulations have progressed and they are still improving [2,3], and currently they represent an innovative area of great scientific interest due to their wide variety of potential applications in biomedicine. Liposomes protect encapsulated molecules against degradation and can be designed to deliver them into specific target cells [4]. Various types of liposomes are used clinically as vehicles for drugs and vaccines [5]. Liposomes can be designed for multiple purposes, including immune modulation to enhance or inhibit a specific response [6].

We recently developed a liposome-based immunotherapy – inspired by the features of apoptotic cells [7] – to re-establish tolerance to self and avoid autoimmune diseases [8]. These liposomes are rich in phosphatidylserine (PS), a phospholipid membrane com-

ponent that is exposed in apoptotic cells and modulates immune responses [9], and are loaded with self-peptides – named autoantigens – as the selective molecule for tolerance induction. PS is recognized by membrane receptors of antigen presenting cells – mainly dendritic cells (DCs) – acting as an ‘eat me’ and ‘tolerate me’ signal, which allows the encapsulated autoantigen to be presented in a tolerogenic manner. Working synergistically, PS-liposomes and encapsulated autoantigens display a big translational potential in pathologies that require the re-establishment of immune tolerance.

Autoimmune diseases are caused by the selective destruction of the host’s own cells by autoreactive T lymphocytes that recognize autoantigens. There are nearly 100 autoimmune diseases, affecting around 3–5% of the population [10]. There is no cure for any of them and their etiology is unknown. Moreover, incidence is increasing in the last

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years [11]. The current therapies for autoimmune diseases are not enough effective and cause side effects, such as immunosuppression. Therefore, antigen-specific, simple and safe approaches to recover self-tolerance are required to arrest autoimmunity and to allow target tissue regeneration. In our previous study, an innovative liposome-based immunotherapy – mimicking apoptotic β cells to induce self-tolerance – arrested autoimmunity in Type 1 diabetes (T1D) [8]. Considering the potential of liposomes as immunomodulators, we aimed to develop PS-liposomes for another autoimmune disease, multiple sclerosis (MS), to validate their tolerogenic power.

MS is a chronic autoimmune disease of the CNS in which several myelin peptides become the target autoantigens of autoreactive T cells [12]. Genetic and environmental factors contribute to the etiology of the disease but the triggering factor is not known. The autoimmune attack involves autoreactive T and B cells [13]. Eighty percent of individuals with MS initially develop a clinical pattern with periodic relapses followed by partial or full remission of the symptoms, called relapsing-remitting MS [14]. This disease is characterized by widespread inflammation, multifocal demyelination and axonal loss. The current treatments are systemic immunointerventions, with important side effects [15]. In this context, PS-liposomes loaded with an autoantigen of the disease, the myelin-oligodendrocyte glycoprotein peptide 40–55 (MOG_{40–55}), have been proved as a potential antigen-specific therapy for MS patients.

For this purpose, we evaluated the *in vivo* efficacy of antigen-loaded PS-liposomes in the animal model of MS, the experimental autoimmune encephalomyelitis (EAE). EAE is an immune-mediated disease that reproduces the main clinical and histopathological characteristics of the human disease. It is induced by the administration of myelin antigens. The most frequent EAE model used is C57BL/6 mice injected with the MOG_{40–55} peptide [16,17], and it has been the major tool to understand the mechanism involved in MS pathogenesis, as well as to test therapies for the human disease. To determine the effect of a therapy in EAE, three different approaches can be used: preventive (before immunization), preclinical (postimmunization) and therapeutic (after the onset of the disease) [17]. Using EAE preclinical approach, we report here the beneficial effect of liposomes rich in PS and loaded with MOG peptide. This work demonstrates that a simple apoptosis-based strategy has the inherent potential of stopping an ongoing autoimmune reaction such as the EAE, validating the immunotherapy and confirming its potential to arrest autoimmunity.

Materials & methods

Mice

Wild-type C57BL/6 inbred mice were purchased from Envigo Rms Spain SL (Sant Feliu de Codines, Barcelona, Spain) and housed at the Animal House facility at the Germans Trias i Pujol Research Institute. Mice were maintained under conventional conditions in a temperature and humidity-controlled room with 12-h light/12-h dark cycle, with standard chow diet (Teklad Global 14% Protein Rodent Diet, Envigo) and water provided *ad libitum*. Only females were used in this study.

Liposomes & peptides

MOG_{40–55} peptide (YRSPFSRVVHLYRNGK, Immunostep, Salamanca, Spain), >95% pure, was chosen because it is a target epitope in MS patients [18]. Liposomes were composed of 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS, Lipoid, Steinhausen, Switzerland) and 1,2-didodecanoyl-sn-glycero-3-phosphocholine (DLPC, Lipoid), and cholesterol (CH, Sigma Aldrich, MO, USA). Liposomes were prepared using the thin film hydration method from a lipid mixture of DOPS, DLPC and CH at 1:1:1.33 molar ratio, respectively, as described [19], under sterile conditions and at a final concentration of 30 mM. Lipids were dissolved in chloroform and the solvent was removed by evaporation under vacuum and nitrogen. The lipids were hydrated with the appropriate buffer (phosphate-buffered saline [PBS], 0.5 mg/ml solution of MOG_{40–55} peptide) and the liposomes obtained were homogenized to 1 μ m by means of an extruder (Lipex Biomembranes, Vancouver, Canada). Encapsulation efficiencies (EE) were calculated according to the equation $EE (\%) = [(C_{\text{peptide, total}} - C_{\text{peptide, out}}) / C_{\text{peptide, total}}] \times 100$, where $C_{\text{peptide, total}}$ is the initial MOG_{40–55} peptide concentration and $C_{\text{peptide, out}}$ is the concentration of nonencapsulated peptide. To measure the $C_{\text{peptide, out}}$, liposome suspensions were centrifuged at 110,000 $\times g$ for 30 min at 10°C. The concentration of nonencapsulated peptide was assessed in supernatants by PIERCE BCA protein assay kit (Thermo Fisher Scientific Inc., IL, USA). In addition to PS-rich liposomes loaded with MOG (PSMOG-liposomes), empty liposomes were generated as controls (PS-liposomes). Particle size distribution and stability – expressed as ζ potential – were measured by dynamic light scattering using Malvern Zetasizer (Malvern Instruments, Malvern, UK) in undiluted samples. Liposome morphology and lamellarity were examined by cryogenic transmission electron microscopy in a JEOL-JEM 1400 microscope (Jeol Ltd., Tokyo, Japan).

DC generation

DCs were differentiated from bone marrow progenitor cells in supplemented RPMI-1640 culture medium

(Biowest, Nuaille, France) containing 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen, CA, USA), 100 U/ml penicillin (Normon SA, Madrid, Spain), 100 mg/ml streptomycin (Reig Jofre, Sant Joan Despi, Spain), 2 mM glutamine (Sigma), 1 mM sodium pyruvate (Gibco), 50 μ M beta-mercaptoethanol (Sigma) and mouse GM-CSF (1000 U/ml; Prospec, Rehovot, Israel), as previously reported [20]. DC purity was assessed by CD11c-PE-Cy7 staining (BD Biosciences, CA, USA) and cell viability was determined by annexin V-PE and 7-AAD staining (BD Biosciences) by flow cytometry (FACS Canto II, BD Biosciences).

Liposome capture by DCs

To determine whether liposome capture by DCs takes place by phagocytosis, DCs were co-cultured with 100 μ M fluorescence labeled PS-liposomes (PS-lipo-OG488; Oregon green 488 DHPE, Invitrogen, CA, USA) during 5 min to 6 h at 37 and 4°C. Cells were extensively washed in PBS to remove all liposomes attached to the cell membrane. Liposome endocytosis was determined by flow cytometry (FACSCanto II, BD Biosciences).

Assessment of DCs phenotype after liposome uptake

DCs were co-cultured with 1 mM empty liposomes (PS-DCs), liposomes loaded with MOG peptide (PSMOG-DCs) or the equivalent amount of MOG peptide (MOG-DCs) during 24 h, and their phenotype was analyzed by flow cytometry (FACSCanto II, BD Biosciences). As controls, DCs were either cultured in basal conditions to obtain immature DCs (iDCs) or with lipopolysaccharide (100 ng/ml; Sigma) for 24 h to obtain mature DCs. DCs were stained with monoclonal antibodies to CD11c-PE-Cy7, CD40-APC, CD86-PE and class II major histocompatibility complex (MHC) I-A/I-E-FITC (BD Biosciences). Corresponding fluorescence minus one staining was used as control. Data were analyzed using FlowJo software (Tree Star, OR, USA).

Prostaglandin E2 quantification

The production of prostaglandin E₂ (PGE₂), a potent immunoregulator responsible for the immunosuppressive mechanism of apoptotic cells, was assessed by ELISA (PGE₂ EIA Kit-Monoclonal; Cayman Chemicals, MI, USA), in supernatants obtained from DC cultures, 24 h after liposome capture. LOD: 80% B/B₀; 15 pg/ml. Sensitivity: 50% B/B₀; 50 pg/ml. Results were expressed as pg of PGE₂/10⁶ cells.

Disease induction & clinical follow-up in the EAE model

For the induction of EAE, C57BL/6 female mice

(Envigo) at 8 weeks of age received subcutaneous injections in both flanks of 50 μ g MOG₄₀₋₅₅ peptide in PBS, emulsified in an equal volume of complete Freund's adjuvant, containing 4 mg/ml of Mycobacterium tuberculosis H37RA (Difco, MI, USA), under ketamine/xylazine at 50 and 5 mg/kg body weight, respectively. In addition, 250 ng of Pertussis toxin (Sigma) were injected intravenously at day 0 and 2. All animals were weighed and examined daily for welfare and clinical signs, according to the following criteria: 0, asymptomatic; 0.5, loss of distal half of tail tone; 1, loss of entire tail tone; 1.5, hind limb weakness; 2, hind limb paralysis; 2.5, hind limb paraplegia; 3, forelimb weakness; 4, quadriplegia; 4.5, severe quadriplegia; 5, quadriplegia; and 6, death. Clinical follow-up analyzes were performed in a blinded manner by two different observers.

Treatment of EAE with liposome-based immunotherapy

Liposomes were administered at day 5 and 9 post immunization (p.i.), after EAE induction but before the disease onset (occurring at day 13 p.i.), to evaluate the potential of the therapy in the prevention of EAE development. At this stage, mice did not show clinical signs of the disease but autoimmune destruction was ongoing. Liposomes were injected intraperitoneally (i.p.) at a dose of 1.75 mg of lipid in 100 μ l of PBS. Mice were treated with liposomes filled with MOG peptide (PSMOG-liposomes, n = 11) and as controls, with empty PS-liposomes (PS-liposomes, n = 10), MOG peptide (MOG, n = 8) or PBS (sham, n = 13). Mice were pooled from two independent experiments.

Analysis of regulatory T cells

Regulatory T cells were assessed in the spleen of PSMOG-liposomes and sham-treated mice at day 15 p.i. This checkpoint was chosen based on the time course of the EAE model. The MOG-specific CD4⁺ T-cell response takes 7 days approximately after EAE induction. Taking into account that liposomes were injected at days 5 and 9 post-immunization, T-cell analysis was performed 6 days later in order to identify the induced tolerogenic mechanisms. Splenocytes were obtained from mice after mechanical disruption and erythrocyte lysis, and analyzed by flow cytometry (FACSCanto II, BD Biosciences). The amount of classical CD4⁺ regulatory T cells (Tregs) was determined after membrane staining (CD3-V450, CD4-APC-Cy7; BD Biosciences and CD25-PE; eBioscience, CA, USA), fixation/permeabilization (FoxP3 fixation/permeabilization Concentrate and Diluent; eBioscience) and intracellular staining (FoxP3-APC; eBioscience). CD4⁺

Table 1. Liposome features.

Liposome type	Lipid concentration (mM)	Diameter (nm)	Polydispersity index (Pdl)	ζ-potential (mV)	Encapsulation efficiency (%)
PSMOG-liposomes	30.01 ± 0.06	861.29 ± 130.49	0.32 ± 0.19	-36.19 ± 5.32	91.53 ± 4.10
PS-liposomes	29.70 ± 0.46	985.33 ± 144.36	0.35 ± 0.05	-35.40 ± 8.44	–

Data presented as mean ± SD.
PSMOG: PS-rich liposome loaded with myelin-oligodendrocyte glycoprotein.

type 1 T regulatory (Tr1) cells were stained with antibodies to CD3-V450, CD4-APC-Cy7, CD49b-FITC and LAG-3-APC (BD Biosciences) [21].

Statistical analysis

The statistical analysis was performed using the Prism 6.0 software (GraphPad software Inc., CA, USA). For comparisons of unpaired data, a nonparametric Mann–Whitney test was used; for paired comparisons, a nonparametric Wilcoxon test was used. Fisher’s exact test was used to compare qualitative variables. For cor-

relation between parameters, Spearman’s test was used. A p-value ≤0.05 was considered significant.

Results

Generation & physicochemical characterization of PS-liposomes loaded with MOG peptide

PS-liposomes were prepared with DOPS/DLPC/CH at 1:1:1.33 molar ratio to present PS on their surface. Liposome preparations were characterized in terms of diameter, polydispersity index (Pdl), stability (ζ-potential) and efficiency of peptide encapsula-

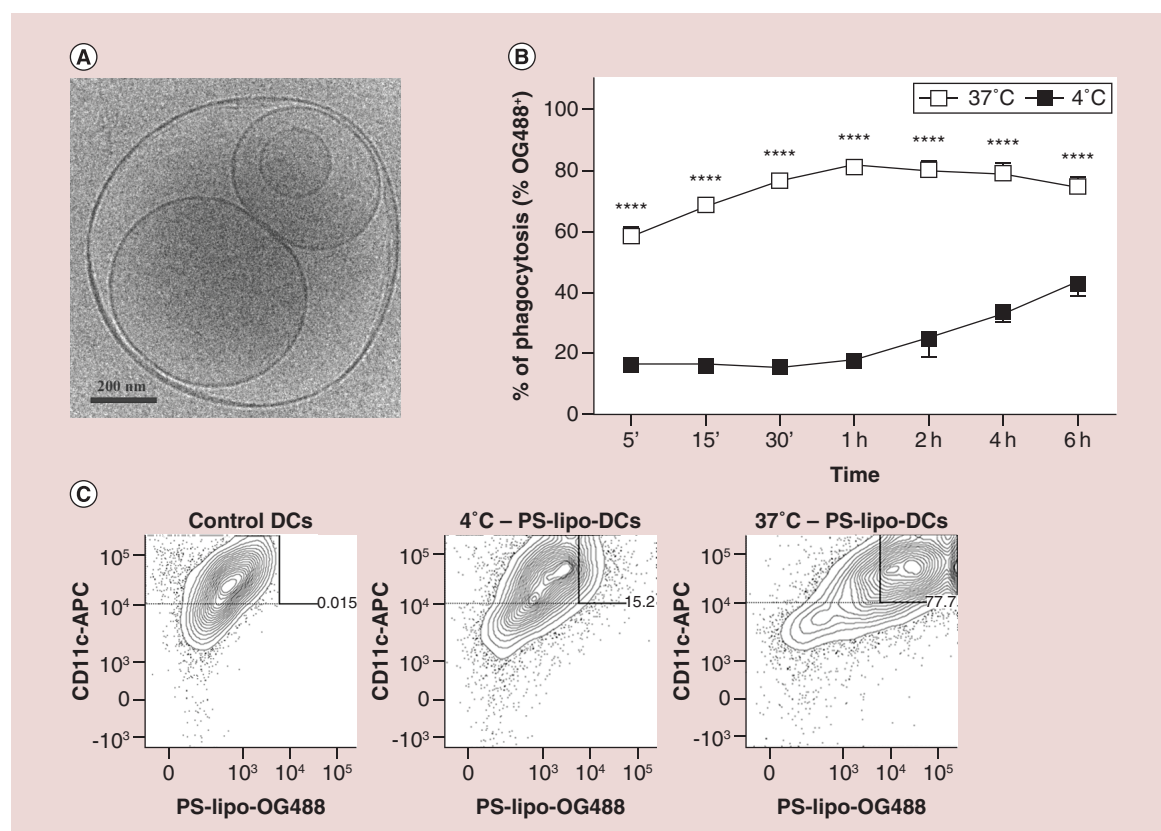


Figure 1. Liposomes are phagocytosed by dendritic cells. (A) Cryogenic transmission electron microscopy images of SMOG-liposomes. Bar = 0.2 μm. (B) Time course of the capture of PS-lipo-OG488 by DCs at 37°C (white squares) and at 4°C (black squares). Results are mean ± SD of three independent experiments (****p < 0.0001, two-way ANOVA). (C) Flow cytometry contour plots of the uptake of PS-lipo-OG488 (OG488+) by DCs (CD11c^{high}). From left to right, control DCs, DCs co-cultured with PS-lipo-OG488 at 4 and 37°C. One representative experiment of three (30 min co-culture) is shown. Percentage of liposome capture (thick line) is referred to CD11c^{high} cell subset (thin line).
DC: Dendritic cell; PS-lipo-OG488: OG488-labeled PS-liposome.

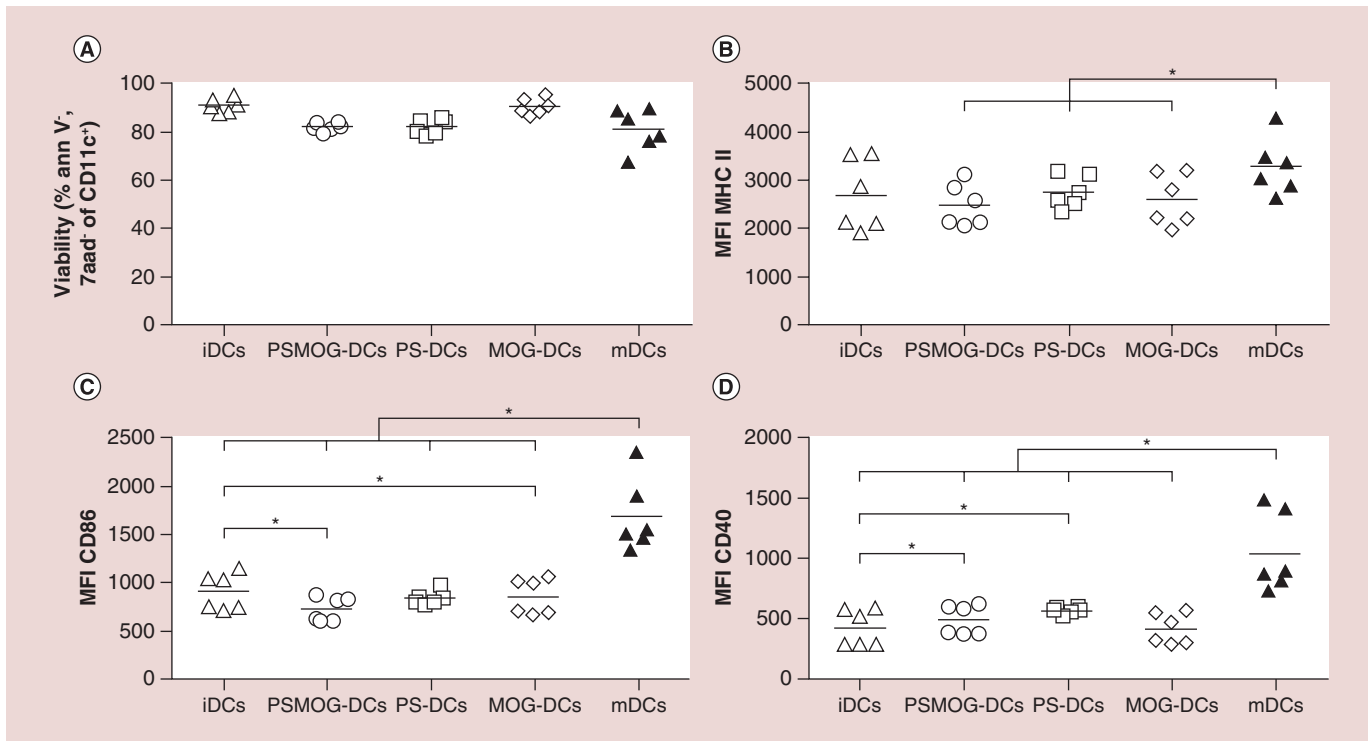


Figure 2. Effect of the capture of phosphatidylserine-liposomes in dendritic cells phenotype. (A) DCs viability assessed by annexin V and 7-AAD staining. White symbols represent iDCs alone (triangles) and after the capture of MOG peptide (MOG-DCs, rhombus), empty PS-liposomes (PS-DCs, squares) or MOG-filled PS-liposomes (PSMOG-DCs, circles), 24 h after culture. Black symbols represent mDCs, after 24-h culture with LPS. Lines show the mean of six independent experiments. (B–D) MFI for MHC Class II, CD86 and CD40 membrane expression, respectively, in DCs before and after liposome capture (white symbols) and after exposure to LPS (black symbols). Lines show the mean of six independent experiments. Comparisons between groups showed significant differences (* $p < 0.05$, nonparametric Wilcoxon test).

DC: Dendritic cell; iDC: Immature DC; LPS: Lipopolysaccharide; mDC: Mature DC; MFI: Median of fluorescence intensity; MHC: Major histocompatibility complex; PS: Phosphatidylserine; PS-DC: DC loaded with empty PS-liposomes; PSMOG-DC: DC loaded with PS-liposomes encapsulating MOG peptide.

tion when necessary (Table 1). The final lipid concentration of PSMOG-liposomes was 30.01 ± 0.06 mM (mean \pm SD) and displayed a mean diameter of 861.29 ± 130.49 nm with a PdI of 0.32 ± 0.19 . ζ -potential measurements revealed a net surface charge of -36.19 ± 5.32 mV, and the mean of MOG peptide encapsulation efficiency was $91.53 \pm 4.10\%$. PSMOG-liposomes presented multivesicular vesicle morphology when cryogenic transmission electron microscopy analysis was performed (Figure 1A). Empty PS-liposomes presented a final lipid concentration of 29.70 ± 0.46 mM, with a mean diameter of 985.33 ± 144.36 nm, a PdI of 0.35 ± 0.05 and a ζ potential of -35.40 ± 8.44 mV.

PS-liposomes are efficiently phagocytosed by DCs

Time course analysis (Figure 1B) revealed that the maximum capture of PS-liposomes by DCs ($81.53 \pm 2.05\%$, mean \pm SD) was achieved after 1 h of co-culture at 37°C . OG488 fluorescence signal at 37°C was higher ($p < 0.0001$) in each checkpoint when compared

with its counterpart at 4°C . The significantly reduced OG488 signal in the experiments performed at 4°C as well as the large diameter of the vesicles (>500 nm) confirms the active process of phagocytosis by which liposomes are captured. Figure 1C shows a representative contour plot of PS-lipo-OG488 uptake by DCs after 30 min of co-culture at 4 and 37°C .

PS-liposomes preserve an immature phenotype in DCs

To assess the tolerogenic potential of PSMOG-liposomes, we analyzed their effect in DCs phenotype. Viability of DCs after the capture of PSMOG-liposomes was always $>80\%$ (Figure 2A). Control DCs, co-cultured with empty PS-liposomes or MOG peptide, behaved similarly to iDCs in terms of viability.

Afterward, the effect of liposome capture in DCs phenotype in terms of costimulation and antigen presentation was examined. Liposome capture did not affect the expression of MHC II in comparison to iDCs (Figure 2B). The expression of CD86 in iDCs decreased

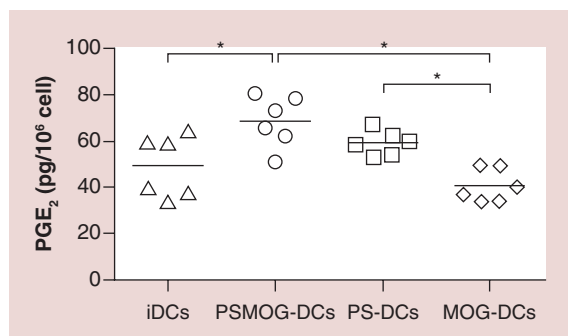


Figure 3. Dendritic cells produce PGE₂ after phagocytosis of PS-rich liposomes loaded with myelin-oligodendrocyte glycoprotein. Quantification of PGE₂ released by iDCs in culture medium (triangles), co-cultured with PSMOG-liposomes (circles), empty PS-liposomes (squares) or MOG peptide (rhombus) after 24 h of culture. Data are represented as pg/10⁶ cells. Lines show the mean of six independent experiments. Comparisons between groups showed significant differences (**p* < 0.05, nonparametric Wilcoxon test). DC: Dendritic cell; iDC: Immature DC; MOG: Myelin-oligodendrocyte glycoprotein peptide 40–55; PGE₂: Prostaglandin E₂; PS: Phosphatidylserine; PSMOG: PS-rich liposome loaded with myelin-oligodendrocyte glycoprotein.

after PSMOG-liposomes capture (*p* < 0.05), and the expression of CD40 in iDCs increased after PS- or PSMOG-liposomes capture (*p* < 0.05) (Figure 2C & D). The expression of CD86, CD40 and MHC class II molecules in DCs after uptake of PSMOG-liposomes was always significantly lower than the observed in mature DCs. These changes suggest that iDCs acquired a semi-mature phenotype after capture of PSMOG-liposomes, a feature of tolerogenic DCs. Control DCs, co-cultured with empty PS-liposomes or MOG peptide, behaved similarly to iDCs in terms of phenotype.

Based on our previous results, PGE₂ production by DCs after PS-liposomes uptake was assessed in culture supernatants. The concentration of PGE₂ was significantly increased in PSMOG-DCs in comparison to iDCs and MOG-DCs (*p* < 0.05), as well as in PS-DCs when compared with MOG-DCs (*p* < 0.05) (Figure 3).

PS-liposomes filled with MOG peptides reduce EAE severity

Mice were treated with two doses of PSMOG-liposomes at days 5 and 9 p.i. to prevent the development of EAE. As expected, all mice from the sham-control group (*n* = 13) developed EAE with a maximum score of 2.83 ± 1.94 (mean \pm SD) (Table 2). The treatment with PSMOG-liposomes (*n* = 11) resulted in a significant reduction of maximum disease score, this being of 1.41 ± 1.59 , in comparison to empty PS-liposomes group (*n* = 10) (*p* < 0.01), whose disease score was 3.90 ± 1.70 , or to MOG peptide group (*n* = 8), whose disease score was 2.38 ± 1.77 . The incidence of the disease was lower in PSMOG-liposomes treated group (45.45%) in comparison to empty PS-liposomes treated (100%, *p* < 0.05) and sham group (92.31%, *p* < 0.05), and slightly lower than the MOG-treated group (75%). Mice treated with PSMOG-liposomes showed a lower cumulative EAE score (14.36 ± 17.89) than mice treated with empty PS-liposomes (45.60 ± 23.61) or sham (42.19 ± 24.99) groups (*p* < 0.01). Moreover, the onset of EAE was at day 16.00 ± 4.56 p.i. in PSMOG-liposomes treated group, 14.80 ± 2.70 in PS-liposomes treated group, 15.50 ± 2.35 in MOG peptide treated group and 13.17 ± 4.73 in sham group. Finally, mice treated with PSMOG-liposomes showed a daily EAE score significantly lower than sham-control group and PS-liposomes treated group (*p* \leq 0.05) (Figure 4A). In addition, mice treated with PSMOG-liposomes did not suffer a decrease in their body weight as mice from PS-liposomes, MOG peptide or sham groups endured (*p* < 0.001) (Figure 4B).

PSMOG-liposomes effect on T-cell subtypes

To test the effect of autoantigen-loaded PS-liposomes administration on regulatory T-cell subsets, intracellular FoxP3 staining was carried out on splenocytes of sham (*n* = 12) and PSMOG-liposomes (*n* = 6) treated animals at day 15 p.i. Looking at CD4⁺ T cells, the treatment with PSMOG-liposomes significantly decreased the percentage of CD25⁺ FoxP3⁺ T cells when compared with sham group, that being of 10.95

Table 2. Clinical features of experimental autoimmune encephalomyelitis in treated and control mice.

Treatment	Sham	MOG	PS-liposomes	PSMOG-liposomes
Incidence	12/13 (92.31%)*	6/8 (75%)	10/10 (100%)*	5/11 (45.45%)
Maximum score	2.83 ± 1.94	2.38 ± 1.77	$3.90 \pm 1.70^{**}$	1.41 ± 1.59
Cumulative score	$42.19 \pm 24.99^{**}$	25.88 ± 21.48	$45.60 \pm 23.61^{**}$	14.36 ± 17.89
Onset day	13.17 ± 4.73	15.50 ± 2.35	14.80 ± 2.70	16.00 ± 4.56

Data are expressed as mean \pm standard deviation. Comparisons between PSMOG-liposomes treated group versus sham and PS-liposomes treated groups showed significant differences (**p* < 0.05, Fisher's Exact test; ***p* < 0.01, Mann-Whitney test). PSMOG-liposome: PS-rich liposome loaded with myelin-oligodendrocyte glycoprotein.

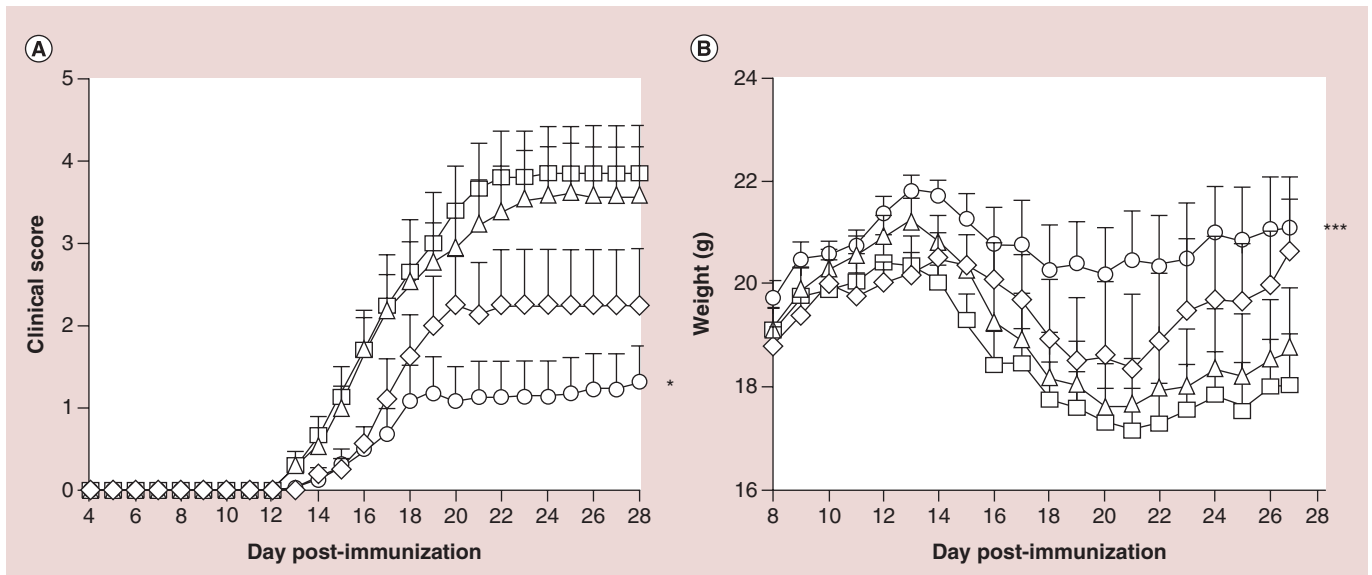


Figure 4. PS-rich liposomes loaded with myelin-oligodendrocyte glycoprotein prevent the development of experimental autoimmune encephalomyelitis. (A) Clinical score of experimental autoimmune encephalomyelitis performed daily for welfare and clinical status as well as neurological signs according to the criteria: 0, asymptomatic; 0.5, loss of distal half of tail tone; 1, loss of entire tail tone; 1.5, hind limb weakness; 2, hind limb paralysis; 2.5, hind limb paraplegia; 3, forelimb weakness; 4, quadriparesis; 4.5, severe quadriparesis; 5, quadriplegia; and 6, death. Follow-up analyzes were performed blindly in mice treated with liposomes containing MOG peptide (PSMOG-liposomes, circles, $n = 11$), empty liposomes (PS-liposomes, squares, $n = 10$), peptide (MOG, rhombus, $n = 8$) or PBS (sham, triangles, $n = 13$). Results are mean \pm standard error of the mean from two independent experiments. Significant differences were found when comparing PSMOG-liposomes group with PS-liposomes and sham groups ($*p \leq 0.05$, nonparametric Mann–Whitney test). (B) Mean weight in experimental autoimmune encephalomyelitis-mice after treatment with liposomes containing MOG peptide (PSMOG-liposomes, circles, $n = 11$), empty liposomes (PS-liposomes, squares, $n = 10$), peptide (MOG, rhombus, $n = 8$) or PBS (sham, triangles, $n = 13$). Results are mean \pm standard error of the mean from two independent experiments. Significant differences were found when comparing PSMOG-liposomes treated group with all control groups ($***p < 0.001$, nonparametric Mann–Whitney test). PSMOG-liposome: PS-rich liposome loaded with myelin-oligodendrocyte glycoprotein.

$\pm 3.48\%$ (mean \pm SD) and $14.69 \pm 2.67\%$, respectively ($p < 0.05$). However, we noted that the percentage of CD25⁺ FoxP3⁺ T cells in PSMOG-liposomes group increased when compared with their sham counterparts, that being of $14.83 \pm 5.47\%$ and $9.65 \pm 2.84\%$, respectively ($p < 0.05$) (Figure 5A). Indeed, the percentage of CD25⁺ FoxP3⁺ T cells in PSMOG-liposomes treated mice correlated inversely with the clinical score at day 15 p.i. (Spearman's $r = -0.9258$, $p < 0.0001$), as shown in Figure 5B, but no correlation was observed between these parameters in sham group (data not shown). Nevertheless, the number of CD25⁺ Foxp3⁺ cells tended to decrease in PSMOG-liposomes group ($6.31 \times 10^5 \pm 2.03 \times 10^5$), when compared with sham group ($11.05 \times 10^5 \pm 0.76 \times 10^5$), although differences are not statistically significant. Regarding the absolute number of CD25⁺ FoxP3⁺ T cells, similar results were obtained in PSMOG-liposomes treated group ($9.13 \times 10^5 \pm 4.58 \times 10^5$) and sham group ($8.95 \times 10^5 \pm 1.71 \times 10^5$). With these results, the expression of CD4⁺ Tr1 cell markers CD49b and LAG-3 was next examined on the splenocytes of the sham and PSMOG-liposomes groups by analyzing their median of fluorescence

intensity. Although there were no statistically significant differences in the expression of CD49b between sham (90.18 ± 11.49 , mean \pm SD) and treated (95.12 ± 13.84) groups, a tendency for this marker to increase in PSMOG-liposomes treated mice was observed (Figure 5C). Supporting this tendency, LAG-3 expression was significantly increased in PSMOG-liposomes treated mice (387.5 ± 88.53) when compared with sham group (304.6 ± 54.41) ($p \leq 0.05$).

Discussion

It is well known that apoptotic cell clearance by phagocytes maintains immunological homeostasis and induces antigen-specific immune tolerance [22,23]. In fact, apoptotic mimicry is a strategy to induce tolerance, lost in autoimmune diseases. In this sense, several approaches have been developed [7,20,24]. We have recently shown that apoptotic mimicry by means of liposomes – rich in PS and encapsulating autoantigens – arrest autoimmunity in T1D [8]. Therefore, we hypothesized that by replacing the encapsulated autoantigen, PS-liposomes would show therapeutic effect for other autoimmune diseases.

We selected MS to assess therapeutic versatility of liposomes. In the present study, we have validated the efficacy of PS-liposomes in the experimental model of the disease. MS is one of the most frequent neurological disorders [25], and epidemiological studies have highlighted an increasing rate of prevalence of the disease. Prevention and cure of MS is not yet possible. Some therapies can slow the progress of the disease and manage the symptoms, but they cause important side effects.

The growing field of micro and nanotechnology has enabled new strategies for MS and other autoim-

mune diseases to be tested in experimental models. Nanoparticles and microparticles have been used to induce T-cell tolerance and as vehicles for anti-inflammatory drugs [26–29], and a few of them have reached the clinic [30]. The relevance of our results relies in the potential of PS-liposomes to develop a platform for the treatment of several autoimmune disorders, using a biomimicry strategy, based on a physiological process such as efferocytosis. In our knowledge, this is the first work in the context of EAE that confirms PS-liposomes as a powerful tool to encapsulate autoantigens, co-delivering a double-signal of tolerance and

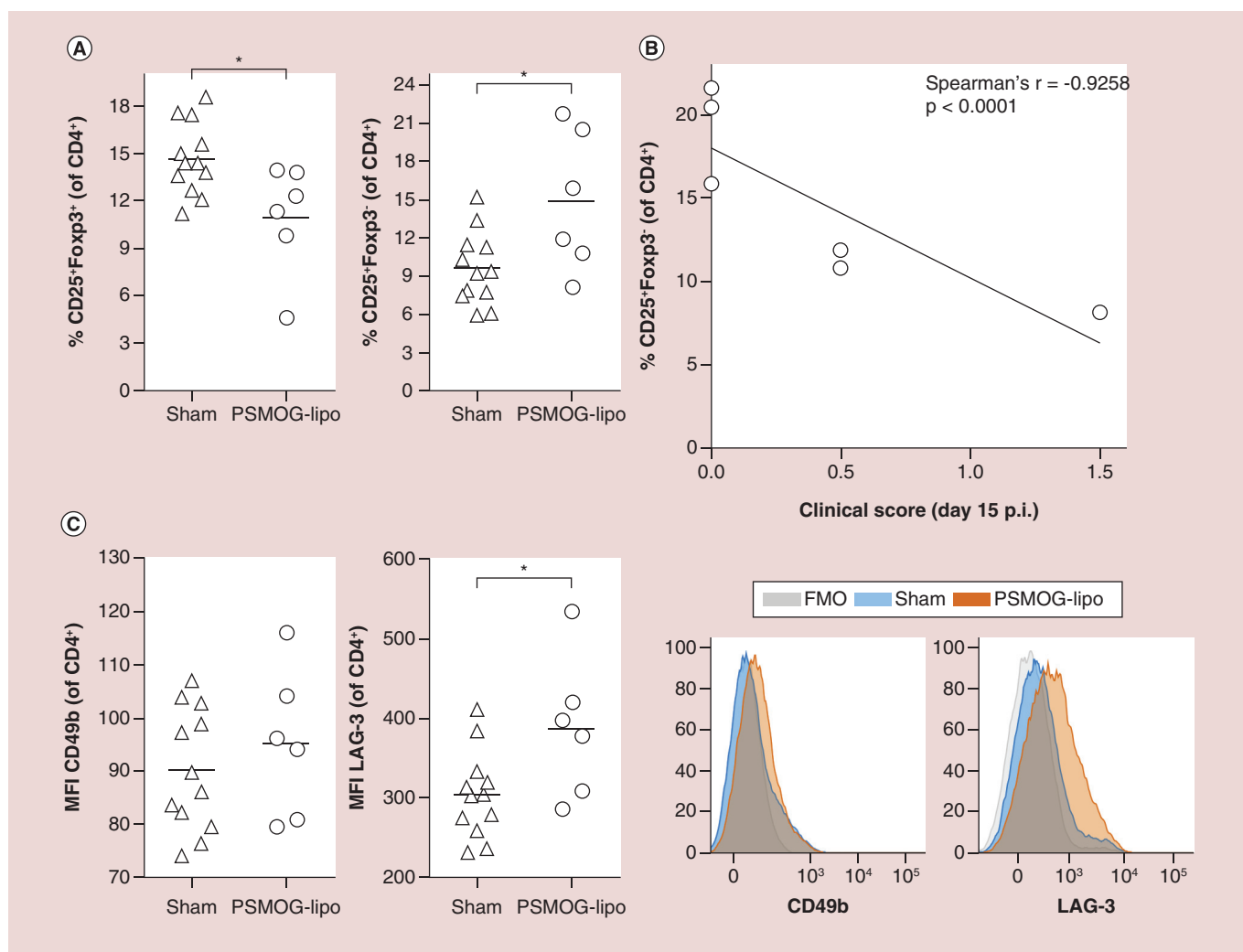


Figure 5. T cell subsets in mice treated with PS-rich liposomes loaded with myelin-oligodendrocyte glycoprotein. (A) Percentage of classical regulatory T cells (CD25⁺ FoxP3⁺ cells gated in CD4⁺ T cells) and CD25⁺ FoxP3⁻ cells (gated in CD4⁺ T cells) in the spleen of PSMOG-liposomes treated mice (circles, n = 6) or sham control group (triangles, n = 12) at day 15 p.i. Lines show the mean of 6–12 mice. Comparisons between groups showed significant differences (p < 0.05, nonparametric Mann–Whitney test). (B) Correlation between clinical score at day 15 p.i. and percentage of CD25⁺ FoxP3⁻ cells (gated in CD4⁺ T cells) in PSMOG-liposomes treated mice (****p < 0.0001, Spearman's correlation analysis). (C) Left panel: MFI of CD49b and LAG-3 membrane expression in CD4⁺ T cells from PSMOG-liposomes treated mice or sham control group at day 15 p.i. Lines show the mean of 6–12 mice. Comparisons between groups showed significant differences (p < 0.05, nonparametric Mann–Whitney test). Right panel: Histogram of one representative experiment is shown for CD49b and LAG3 expression in mice treated with PSMOG-liposomes (orange) or sham (blue). FMO: Fluorescence minus one; MFI: Median of fluorescence intensity; P.i.: Post immunization.

specificity to arrest autoimmune reactions in a synergistic, effective and safe manner. Liposomes provide an effective tolerogenic-signal induction with additional advantages. They are biocompatible, completely biodegradable, nontoxic, nonimmunogenic, suitable for encapsulation of peptides with varying solubility and low cost [31]. Moreover, they protect the encapsulated drug from the external environment and exhibit flexibility to be coupled with site-specific ligands (e.g., PS and its receptor in DCs).

Various types of liposomes are being used as vehicles [32] for anticancer drugs, vaccines and anti-inflammatory agents [2,33,34]. In this sense, liposomes have been used for the treatment of MS but PS was not included in their composition [35]. PS is the main 'eat me' and 'tolerate me' signal of the apoptotic cell membrane, which allows recognition and phagocytosis by antigen presenting cells such as DCs [36]. Apoptotic cell signaling prevents maturation of DCs and promotes autoantigen presentation in a tolerogenic manner, to induce specific tolerance rather than autoimmunity [7]. Conversely, a high rate of apoptosis and/or defects in their clearance contribute to autoimmunity, since apoptotic cells turn into secondary necrotic cells, releasing proinflammatory signals that enable maturation of DCs and immunogenic presentation of autoantigens. In this sense, neuronal apoptosis is induced in cultures exposed to cerebrospinal fluid from MS patients [37], pointing to a relevant role of apoptosis in the disease progression. These data fit well with the herein reported effect of PSMOG-liposomes in the re-establishment of self-tolerance in the EAE model.

The morphological and physicochemical features of the liposomes are key to provide them with this tolerogenic potential. To validate the liposome effect reported in experimental T1D in MS, we designed EAE-adapted liposomes to be similar to those used for T1D. In this work, we demonstrate the capacity of this already-published lipid composition to prevent a completely different autoimmune disease by replacing the relevant autoantigen in T1D with the corresponding peptide for EAE and keeping lipid concentration, diameter and charge. This conceptual innovation allows us to consider this strategy as a potential platform for the reestablishment of immunological tolerance. *In vitro* experiments demonstrate that liposomes are phagocytosed by DCs from another experimental model (EAE) inducing the same phenotypic and functional changes in DCs than in the T1D model [8].

In vitro experiments revealed crucial immunological consequences of the capture of PSMOG-liposomes by iDCs. First, liposomes were rapidly, actively and safely captured through phagocytosis by DCs, as demonstrated by co-culture experiments at 37 and 4°C [38,39].

Endocytic processes involve the internalization of large-sized particles or a large volume of the extracellular bulk. These are termed phagocytosis and macropinocytosis, respectively: *phagocytosis* refers to the transport of large particles (>250 nm in diameter) by immune system specialized cells (macrophages, DCs and neutrophils), while *pinocytosis* refers to ingestion of fluids and solutes (about 100 nm in diameter) by mammalian cells [40]. In this sense, the large size of the here reported liposomes – diameter greater than 500 nm – and the presence of PS as an 'eat-me' signal promote active phagocytosis by DCs [8,41]. Second, after liposome engulfment, DCs increased CD40 membrane expression and decreased CD86, and maintained low membrane expression of MHC class II expression, features of tolerogenic DCs. Interestingly, PSMOG-liposomes uptake induced the secretion of PGE₂, an essential mediator in the maintenance of tolerance after efferocytosis [42]. These results are in agreement with our previous study, using specific PS-liposomes for T1D.

Importantly, liposome treatment was successful at arresting autoimmunity *in vivo* and greatly ameliorating clinical symptoms in treated mice in terms of severity, incidence and disease onset. As expected, no effect was observed when mice received empty PS-liposomes. As described from previous studies [43], treatment with MOG peptide resulted in an amelioration of the disease, and it was in an antigen-specific manner [44]. However, the effect of MOG peptide appeared to be minor than the induced by PSMOG-liposomes, which reflect the joint action of both PS and MOG. Also, combined therapy with MOG peptide and other compounds such as vitamin D or Rapamycin displays synergisms in ameliorating EAE [45,46]. Here we show the antigen specificity of the treatment, as empty PS-liposomes do not provide protection in front EAE symptoms whereas treatment with liposomes filled with MOG peptide clearly resulted in a strong protection from disease. This immunotherapy is safe and even more biologically effective than the treatment with MOG peptide because PS-liposomes mimic apoptotic cells, thus providing specificity and tolerogenic signals to DCs. These results are consistent with previous data obtained in T1D studies, showing a requirement for β -cell autoantigens in liposomes to arrest autoimmunity to islet cells [8].

The mechanism of action of the herein reported immunotherapy should be grounded on the promotion of regulatory mechanisms by DCs after liposome capture. The decrease in frequency of CD25⁺ FoxP3⁺ CD4⁺ T cells as well as in absolute numbers (although nonstatistically significant) is consistent with an increase in frequency of CD25⁺ FoxP3⁻ CD4⁺ T cells.

However, absolute numbers of CD25⁺ FoxP3⁻ CD4⁺ T cells are not altered by liposomes, suggesting that additional regulatory mechanisms may be involved. These results are in line with those obtained in experimental autoimmune T1D [8]. In fact, CD25⁺ FoxP3⁻ CD4⁺ T-cell frequency is higher in treated mice with minor EAE clinical score, so it is reasonable to speculate that these cells have a regulatory function and can belong to a subset of antigen-specific regulatory T lymphocytes. For instance, we cannot rule out an increase in antigen-specific Tr1 cells after PSMOG-liposomes treatment, based on the CD49b and LAG-3 expression profile [21], as reported in other nanotherapies aimed to restore self-tolerance [8,29]. LAG-3 marker is significantly increased in mice treated with PSMOG-liposomes and, although statistically non-significant, CD49b shows a tendency to increase in treated mice. In this sense, LAG-3 expression is sufficient to confer regulatory activity in CD4⁺ T cells [47], upholding the possible generation of Tr1 with the described immunotherapy.

Altogether our results highlight the potential of a PS-liposomes platform for the prevention and reversion of autoimmune diseases, with advantages in terms of production feasibility, stability, safety, biocompatibility, costs and customization for different diseases, as long as the autoantigen is identified. The here reported work validates PS-liposomes as a powerful tool to encapsulate autoantigens, co-delivering a double-signal of tolerance and specificity to arrest autoimmunity in a synergistic and effective manner.

Conclusion

PS-liposomes encapsulating self-peptides are sphere-shaped microvesicles designed to achieve tolerogenic delivery of autoantigens into antigen presenting cells in order to arrest autoimmunity. PS-liposomes loaded with a MS autoantigen (MOG) were prepared and tested in an experimental model of the disease, the EAE-mouse model. PSMOG-liposomes were efficiently captured by DCs, inducing a tolerogenic phenotype, and ceasing the autoimmune reaction in EAE-mice, in the same way that PS-liposomes customized for T1D do. This work validates PS-liposomes as a powerful tool for the re-establishment of tolerance and for the treatment of different autoimmune diseases.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2016-0410

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Summary points

Background

- The prevalence of autoimmunity is on the rise, and there is no cure for any autoimmune disease, caused by the loss of tolerance to self.
- One of the mechanisms to maintain self-tolerance is the efficient removal of apoptotic cells. For this reason, a liposome-based antigen-specific immunotherapy was generated.
- Liposomes that mimic apoptotic β cells prevent Type 1 diabetes through specific and definitive re-establishment of tolerance.

Aim

- Having proved that liposomes rich in phosphatidylserine (PS) and loaded with autoantigens were effective for Type 1 diabetes, our aim was to validate the immunotherapy in other autoimmune disease: multiple sclerosis.

Results

- PS-Liposomes loaded with a multiple sclerosis autoantigen are efficiently phagocytosed by dendritic cells and induce tolerogenic features in dendritic cells.
- After post immunization administration, MOG-loaded PS-liposomes reduce the incidence and severity of experimental autoimmune encephalomyelitis, and delay the onset.
- The arrest of the autoimmune attack correlates with an increase in the CD25⁺ FoxP3⁻ CD4⁺ T-cell subset.

Conclusion

- This is the first work in the context of experimental autoimmune encephalomyelitis that confirms PS-liposomes as a powerful tool to encapsulate autoantigens, co-delivering a double-signal of tolerance and specificity to arrest autoimmunity in a synergistic, effective and safe manner. Autoantigen-loaded PS-liposomes are candidates for immunotherapy to induce self-tolerance, with high potential to operate as a platform for autoimmune diseases.

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