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► To cite this version:

Lidia Almenara-Fuentes, Silvia Rodriguez-Fernandez, Estela Rosell-Mases, Katerina Kachler, Axel You, et al. A new platform for autoimmune diseases. Inducing tolerance with liposomes encapsulating autoantigens. *Nanomedicine: Nanotechnology, Biology and Medicine*, 2023, pp.102635. 10.1016/j.nano.2022.102635 . hal-03885962

HAL Id: hal-03885962

<https://hal.science/hal-03885962v1>

Submitted on 17 Nov 2023

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A new platform for autoimmune diseases. Inducing tolerance with liposomes encapsulating autoantigens

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Revised 20 September 2022

Abstract

Autoimmune diseases (AIDs) are caused by the loss of self-tolerance and destruction of tissues by the host's immune system. Several antigen-specific immunotherapies, focused on arresting the autoimmune attack, have been tested in clinical trials with discouraging results. Therefore, there is a need for innovative strategies to restore self-tolerance safely and definitively in AIDs. We previously demonstrated the therapeutic efficacy of phosphatidylserine (PS)-liposomes encapsulating autoantigens in experimental type 1 diabetes and multiple sclerosis. Here, we show that PS-liposomes can be adapted to other autoimmune diseases by simply replacing the encapsulated autoantigen. After administration, they are distributed to target organs, captured by phagocytes and interact with several immune cells, thus exerting a tolerogenic and immunoregulatory effect. Specific PS-liposomes demonstrate great preventive and therapeutic efficacy in rheumatoid arthritis and myasthenia gravis. Thus, this work highlights the therapeutic potential of a platform for several autoimmunity settings, which is specific, safe, and with long-term effects.

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Keywords: Immunotherapy; Autoimmune diseases; Nanomedicine; Myasthenia gravis; Rheumatoid arthritis; Preclinical mouse models

Background

Autoimmune diseases (AIDs) are chronic diseases caused by the lack of self-tolerance and the destruction of tissues by the host's immune system. There are about 100 autoimmune disorders, including type 1 diabetes (T1D), multiple sclerosis (MS), coeliac disease, rheumatoid arthritis (RA), and myasthenia gravis

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(MG). AIDs are a major health concern in developed countries and their incidence is increasing.¹

Standard treatments for AIDs usually consist of immune-suppressive and non-disease-specific drugs, associated with long-term side effects. To directly tackle the root cause of these disorders, several antigen-specific immunotherapies with the potential to restore selectively the breach of tolerance to auto-antigens have been tested in clinical trials, but the results have been rather discouraging. Therefore, there is a clear need for innovative therapies to restore self-tolerance safely and definitively in AIDs. Antigen-specific therapies are precision strategies aiming at inducing an immunoregulatory response capable of arresting the autoimmune attack and, consequently, reversing AIDs.²

We previously demonstrated the efficacy of a liposome-based immunotherapy in AIDs. This strategy takes advantage of efferocytosis –apoptotic cell clearance–, a conserved mechanism of immunological tolerance induction.^{3,4} When apoptosis occurs, phosphatidylserine (PS) – a phospholipid kept in the inner leaflet of the lipid bilayer in viable cells – translocates to the outer leaflet and interacts with phagocytes, triggering efferocytosis and enforcing the cell-tolerance message. After efferocytosis, phagocytes promote tolerance to the self-antigens harvested from apoptotic cells. Based on this homeostatic mechanism, we devised an immunotherapy consisting of auto-antigen-loaded PS-rich liposomes mimicking apoptotic cells. As a result, PS-liposomes encapsulating autoantigens induced a binary signal of tolerance and antigen-specificity through the generation of tolerogenic dendritic cells (tolDCs) and expansion of regulatory T (Treg)-cells in experimental T1D – non-obese diabetic (NOD) mouse – and MS – experimental autoimmune encephalomyelitis (EAE), hindering the development of their respective diseases.^{5,6} Advancing in the preclinical phase, the immunomodulatory capacity of these liposomes was confirmed in DCs from patients with T1D, which switched to a tolerogenic profile in terms of transcriptome, phenotype and functionality.^{7,8}

These data describe a platform for tolerance induction that is specific, biocompatible, and with long-term effects.⁹ Here, we determine the biodistribution of the therapy and the cells involved in their tolerogenic effect which includes every cellular agent in the immune response from phagocytes to effector and regulatory B (Breg)- and Treg-cells. To strengthen the potential of this immunotherapy, RA and MG specific nanoparticles were generated, demonstrating great preventive and therapeutic efficacy in preclinical models. In summary, these results highlight the therapeutic potential of liposomes for tolerogenic autoantigen delivery and long-term abrogation of autoreactive immune responses.

Methods

Detailed methods can be found in the Supplementary data section.

Liposome manufacturing

Several autoantigenic peptides were selected based on their reported relevance in AIDs. Briefly, the peptides – murine 2.5mi,

human Vimentin and human mutated citrullinated vimentin (MCV), murine myelin oligodendrocyte glycoprotein (MOG35-55) and human acetylcholine receptor (AChR146-162), human insulin and human methylated bovine serum albumin (mBSA) – were encapsulated within liposomes composed of 1,2-dioleoyl-sn-glycero-3-phospho-L-serine, 1,2-dimyristoyl-sn-glycero-3-phosphocholine and cholesterol. Liposomes were manufactured in Ahead Therapeutic's cleanroom (l'Arboç, Spain) using the thin-film hydration method and characterized as described.⁵

Mice

Wild-type NOD mice and NOD.BDC2.5 mice were bred in the Centre for Comparative Medicine and Bioimage (Badalona, Spain) under specific-pathogen-free conditions. Wildtype C57BL/6NRj were used for establishing the RA model at the University of Erlangen-Nürnberg (Germany). The experimental autoimmune MG (EAMG) model was established with C57BL/6 mice at Sorbonne University (France). All experiments were performed in full compliance with the recommendations in the Declaration of Helsinki for animal experimental investigation and in the Principles of Laboratory Animal Care by the National Institute of Health. The protocols were approved by the animal ethical committees of the participating institutions' governments.

Liposome biodistribution in NOD mice

Prediabetic NOD mice were treated with 200 µl intraperitoneally (i.p.), 50 µl intradermally (i.d.) or 100 µl intravenously (i.v.) of DOPE-AF750-PS-liposomes. *In vivo* and *ex vivo* imaging of selected organs was performed with Pearl Impulse system (LI-COR). Signal was expressed as Relative Fluorescence Units (RFU) per grams of tissue of each organ.

In vivo capture of fluorescent liposomes in NOD mice

Prediabetic female NOD mice were treated with 200 µl i.p. or 100 µl i.v. of NBD-PS-liposomes or NBD-PSIns(h)-liposomes. At 6 h and 24 h post-injection, splenocytes were obtained and labeled prior to FACS LSR Fortessa (BD Biosciences) acquisition – additional information in Table 1 and Supplementary Table 1. Corresponding Fluorescence Minus One (FMO) staining was used as a control and FlowJo software (Tree Star) was used for the data analysis.

In vitro liposome capturing assessment by flow cytometry in NOD's splenocytes

Splenocytes from female NOD mice were incubated with 1 mM NBD-PS-liposomes or NBD-PSIns(h)-liposomes overnight. Interleukin (IL)-10⁺ secreting cells were stained extracellularly with monoclonal antibodies and the APC Mouse IL-10 Secretion Assay Detection Kit (Miltenyi Biotech) – additional information in Table 1 and Supplementary Table 1. Cells were acquired using FACS LSR Fortessa and FlowJo software was used for the data analysis.

Table 1
Immune cell subsets determined by flow cytometry analysis.

Subset name	Phenotype
T cells	CD3 ⁺
Foxp3 ⁺ CD4 ⁺ Treg cells	CD3 ⁺ CD4 ⁺ Foxp3 ⁺
Foxp3 ⁺ CTLA4 ⁺ CD4 ⁺ Treg cells	CD3 ⁺ CD4 ⁺ Foxp3 ⁺ CTLA4 ⁺
Foxp3 ⁺ CD8 ⁺ Treg cells	CD3 ⁺ CD8 ⁺ Foxp3 ⁺
Foxp3 ⁺ CTLA4 ⁺ CD8 ⁺ Treg cells	CD3 ⁺ CD8 ⁺ Foxp3 ⁺ CTLA4 ⁺
TR1 cells	CD3 ⁺ CD4 ⁺ Foxp3 ⁻ LAG3 ⁺ CD49b ⁺
Anergic CD4 ⁺ T cells	CD3 ⁺ CD4 ⁺ Foxp3 ⁻ CD73 ⁺ FR4 ⁺
Anergic CD8 ⁺ T cells	CD3 ⁺ CD8 ⁺ Foxp3 ⁻ CD73 ⁺ FR4 ⁺
Exhausted CD4 ⁺ T cells	CD3 ⁺ CD4 ⁺ PD-1 ⁺
Exhausted CD8 ⁺ T cells	CD3 ⁺ CD8 ⁺ PD-1 ⁺
B cells	CD19 ⁺
CD1d ^{high} CD5 ⁺ B cells	CD19 ⁺ CD1d ^{high} CD5 ⁺
B1a B cells	CD19 ⁺ CD5 ⁺ CD43 ⁺
B1b B cells	CD19 ⁺ CD5 ⁻ CD43 ⁺
Marginal Zone (MZ) B cells	CD19 ⁺ CD21 ^{high} CD23 ⁻
Foxp3 ⁺ B cells	CD19 ⁺ Foxp3 ⁺
CD1d ⁺ LAG3 ⁺ B cells	CD19 ⁺ CD1d ⁺ LAG3 ⁺
Conventional DCs (cDCs)	CD11c ⁺ MHC-II ⁺ CD205 ⁺ CD8a ⁺
Plasmacytoid DCs (pDCs)	CD11c ⁺ MHC-II ⁺ B220 ⁺
Macrophages (Mφ)	F4/80 ⁺
Osteoclast progenitor cells (OCPs)	RANK ⁺ MCSFR ⁺ CD11b ⁺ Ly6G ⁻

Treg, regulatory T; DCs, dendritic cells.

Analysis of Treg-cell subsets after liposome treatment in NOD.BDC2.5 mice

Female NOD.BDC2.5 mice were treated with 100 µl i.v. or 50 µl i.d. of empty PS-liposomes or PS2.5mi-liposomes and euthanized at day 4 or day 13 post-injection. Splenocytes were obtained and labeled extracellularly with several antibodies and intracellularly with Foxp3 – additional information in Table 1 and Supplementary Table 1. Cells were acquired using FACS LSR Fortessa and FlowJo software was used for the data analysis.

RA induction and liposome treatment in the experimental model

A modified antigen-induced arthritis (AIA) model, which mimics the anti-MCV-antibody-mediated immune response and periarticular bone loss in RA, was used.¹⁰ For disease induction, 12-week-old C57BL/6NRj female mice were immunized by subcutaneously with 0.1 mg MCV (provided by Holger Bang) and 0.1 mg mBSA, dissolved in PBS and emulsified with Complete Freund Adjuvant (CFA). After 3 days, mice were injected i.p. with 200 µl of PS-liposomes, PSVim-liposomes or PSMCV-liposomes. On day 7 after the primary immunization, mice were immunized a second time by intraarticular injection. Knee swelling was measured on days 7–14 using a caliper and the experiment ended on day 14 after the primary immunization.

Bone marrow analysis in the RA model

Bone marrow cells were flushed from femoral bones using PBS, and cell suspension labeled prior to Cytoflex S flow cytometer acquisition – additional information in Table 1 and

Supplementary Table 1. Data were analyzed using Kaluza 2.1 (Beckman Coulter).

Histological analysis in the RA model

Knee joints and tibial bones were fixed, decalcified, and embedded in paraffin. Tissue was sectioned prior to staining with hematoxylin and eosin (H&E) for inflammation quantification, or with tartrate-resistant acid phosphatase staining (TRAP, Leukocyte Acid Phosphatase Kit, Merck) to determine bone erosion and osteoclast number, and red on an Axio Lab.A1 microscope.

MG induction and liposome treatment

6–8-week-old C57BL/6 female mice were immunized with purified T-AChR, emulsified with an equal volume of CFA and supplemented with Mycobacterium. After 22 days, mice were boosted with T-AChR/CFA or just CFA (control group). Clinical signs were evaluated throughout the study and the Global Clinical Score (GCS) was graded on 9 (Supplementary Table 2). Fourteen days after boost, EAMG mice were separated into three homogeneous groups (based on the GCS) including mice with or without clinical signs. Mice were non-treated (CFA control group) or treated i.p. with 200 µl empty PS-liposomes, PSmMOG-liposomes or PSACHR-liposomes. Blood samples were taken on days 35, 50, 66, 81 and 95 to determine anti-TAChR antibodies by ELISA.

Statistical analysis

Statistical analysis was performed using the Prism 9.0 software (GraphPad Software Inc.). For comparison between unpaired data, a parametric two-tailed Student's t-test or non-parametric Mann-Whitney test was used. For comparison among multiple groups, one-way ANOVA or two-way ANOVA with the corresponding multiple comparison test were performed. P-value <0.05 was considered significant.

Results

PS-liposomes are optimally generated for different AIDs

We previously demonstrated that T1D and MS autoantigens were efficiently encapsulated in PS-liposomes.^{5,6} Here we show that PS-liposomes encapsulating other autoantigens such as MCV or AChR peptides can be generated for RA and MG treatment, respectively. These particles always had a hydrodynamic size larger than 800 nm. Liposome preparations were characterized in terms of intensity weighted mean hydrodynamic size (nm), PdI, stability (ζ-potential) and efficiency of peptide encapsulation (Table 2).

Fluorescent-labeled PS-liposomes are distributed to target organs and captured by phagocytes in NOD mice

T1D is caused by the immune-mediated destruction of insulin-producing β-cells. Patients require exogenous insulin administration for life, a treatment not exempt from short and long-term complications.¹¹ Autoantigen-loaded PS-liposomes

Table 2
Physicochemical features of PS-liposomes.

PS-liposomes batch	Hydrodynamic size (nm)	Polydispersity index	ζ -potential (mv)	Encapsulated peptide	Encapsulation efficiency (%)
DOPE-AF750-PS-liposomes (empty)	720.50 \pm 6.4	0.28 \pm 0.04	-26.3 \pm 0.6	-	-
NBD-PS-liposomes (empty)	788.8 \pm 282.4	0.27 \pm 0.13	-26.5 \pm 3.9	-	-
NBD-PSIns(h)-liposomes	894.7 \pm 314.1	0.27 \pm 0.15	-26.7 \pm 3.8	Human insulin	84.6 \pm 18.9
PS-liposomes (empty)	1104.8 \pm 256.7	0.27 \pm 0.09	-28.8 \pm 5.2	-	-
PS2.5mi-liposomes	1191	0.14	-27.1	Murine 2.5mi	25.9
PSVim-liposomes	940	0.39	-35.9	Unmodified human vimentin	71.5
PSMCV-liposomes	1037	0.24	-38.2	Human mutated citrullinated vimentin	65.9
PSmMOG-liposomes	1761	0.32	-27.0	Murine myelin oligodendrocyte glycoprotein (MOG ₃₅₋₅₅)	96.0
PSAChR-liposomes	1177	0.21	-28.9	Human acetylcholine receptor (AChR146-162)	47.3

Data presented as mean \pm SD when two or more liposomes' batches were used.

immunotherapy was proven effective in arresting autoimmunity in NOD mice.⁵ To gain mechanistic insight, we first studied PS-liposomes biodistribution. Thus, empty fluorescent-labeled AF750-PS-liposomes were administered by i.p., i.d. and i.v. routes to prediabetic NOD mice, and fluorescent signal in each organ was evaluated after 1-, 6- and 24 h post-administration (Fig. 1, Supplementary Fig. 1). Although the fluorescent signal is not comparable between routes because each route allowed for a different maximal administered volume, the i.p. route achieved fluorescence values between 185.5 and 112,541 RFU/g of tissue at 1 h after injection; the i.d. route, 34.55 and 2960 RFU/g, and the i.v. route, 187.5 and 37,552 RFU/g. Also, whereas the i.v. route showed peak values 1 h after injection, the i.p. and i.d. route showed a slower biodistribution with peak fluorescence values being reached 6 h after injection in some organs. Nevertheless, the 1 h and 6 h checkpoints showed considerably higher levels of fluorescence than the 24 h checkpoint in all routes. Thus, in the i.p. route, considerable levels of fluorescence were gathered in the bladder, liver, mediastinal lymph nodes (MDLN), pancreas, pancreatic lymph nodes (PLN), perigonadal adipose tissue (PAT) and spleen. In the i.d. route, fluorescence predominantly accumulated in axillary lymph nodes (LN), bladder, kidney, lung, MDLN, pancreas, PLN, PAT and stomach. In the i.v. route, fluorescence specially gathered in the liver, lung, MDLN, spleen and stomach.

Then, we interrogated which immune cells interacted with PS-liposomes *in vivo*. Thus, NOD mice received a single dose i.p. or i.v. of fluorescent-labeled NBD-PS-liposomes loaded with human insulin (NBD-PSIns(h)-liposomes). The spleen was harvested 6 h and 24 h post-administration. The splenocytes viability was 98.8 \pm 0.23 (mean \pm SEM, data not shown). In Fig. 2, regarding the i.p. route, at the time-point of 6 h, the immune cells with the highest percentage of NBD positivity were macrophages (25.5 \pm 9.4 %), followed by conventional DCs (cDCs) (14.5 \pm 4.0 %), as expected due to their phagocytic function. Plasmacytoid DCs (pDCs) also showed NBD signal (8.9 \pm 3.3 %), as well as a low percentage of B and T-cells (1.4 \pm 0.5 % and 0.9 \pm 0.3 %, respectively). At 24 h, the signal had statistically decreased in macrophages, and tended to decrease in cDCs and pDCs. Similarly, in the i.v. route, 6 h after liposome adminis-

tration, the highest levels of fluorescence were exhibited by cDCs (8.6 \pm 2.1 %) and macrophages (8.3 \pm 1.4 %) and were maintained after 24 h in cDCs (9.6 \pm 2.3 %). Finally, NBD-PS-liposomes interacted similarly with immune cell subsets in the spleen (Supplementary Fig. 2), and no differences were found among empty and loaded NBD-PSIns(h)-liposomes at any time point nor administration route.

PS-liposomes interact with Breg-cell subsets and promote IL-10 secretion

Because B-cells play an important role as antigen-presenting cells (APCs) in T1D, we determined the tolerogenic effect of PS-liposomes in B-cells. Thus, splenocytes from NOD mice were co-cultured with NBD-PSIns(h)-liposomes. According to our previous experience, the splenocytes viability after the overnight culture with NBD-PSIns(h)-liposomes was 70.0 \pm 2.6 % (mean \pm SEM) (Fig. 3a), discarding any toxic effect. Also, among all B lymphocytes, 34.8 \pm 2.0 % showed NBD fluorescence. When we dissected NBD fluorescence in the different B-cell subsets with described regulatory potential, CD5⁺CD1d^{high} cells showed 48.1 \pm 2.3 % of NBD signal (with respect to the whole CD5⁺CD1d^{high} subset); NBD⁺ B1a B lymphocytes, 58.7 \pm 1.4 % of all B1a B lymphocytes; NBD⁺ B1b B lymphocytes, 24.8 \pm 1.5 % of all B1b B lymphocytes, and NBD⁺ marginal zone (MZ) B-cells, 67.5 \pm 0.4 % of all MZ B-cells (Fig. 3b). To demonstrate their regulatory function after treatment, positively-secreting IL-10 cells were found in each subset: 13.1 \pm 1.4 % of CD5⁺CD1d^{high} NBD⁺ cells, 16.9 \pm 1.9 % of B1a NBD⁺ cells, 1.5 \pm 0.8 % of B1b NBD⁺ cells, and 13.4 \pm 0.9 %, of MZ NBD⁺ cells (Fig. 3c). Despite differences are not significant, the IL-10 secretion showed a biological tendency to be higher in NBD-fluorescent B-cells when compared to non-fluorescent B-cells for each subset.

Specific PS-liposomes induce a T-cell immunoregulatory profile in NOD.BDC2.5 mice

To determine the tolerogenic effect of the immunotherapy, NOD.BDC2.5 mice were treated with PS2.5mi-liposomes or PS-

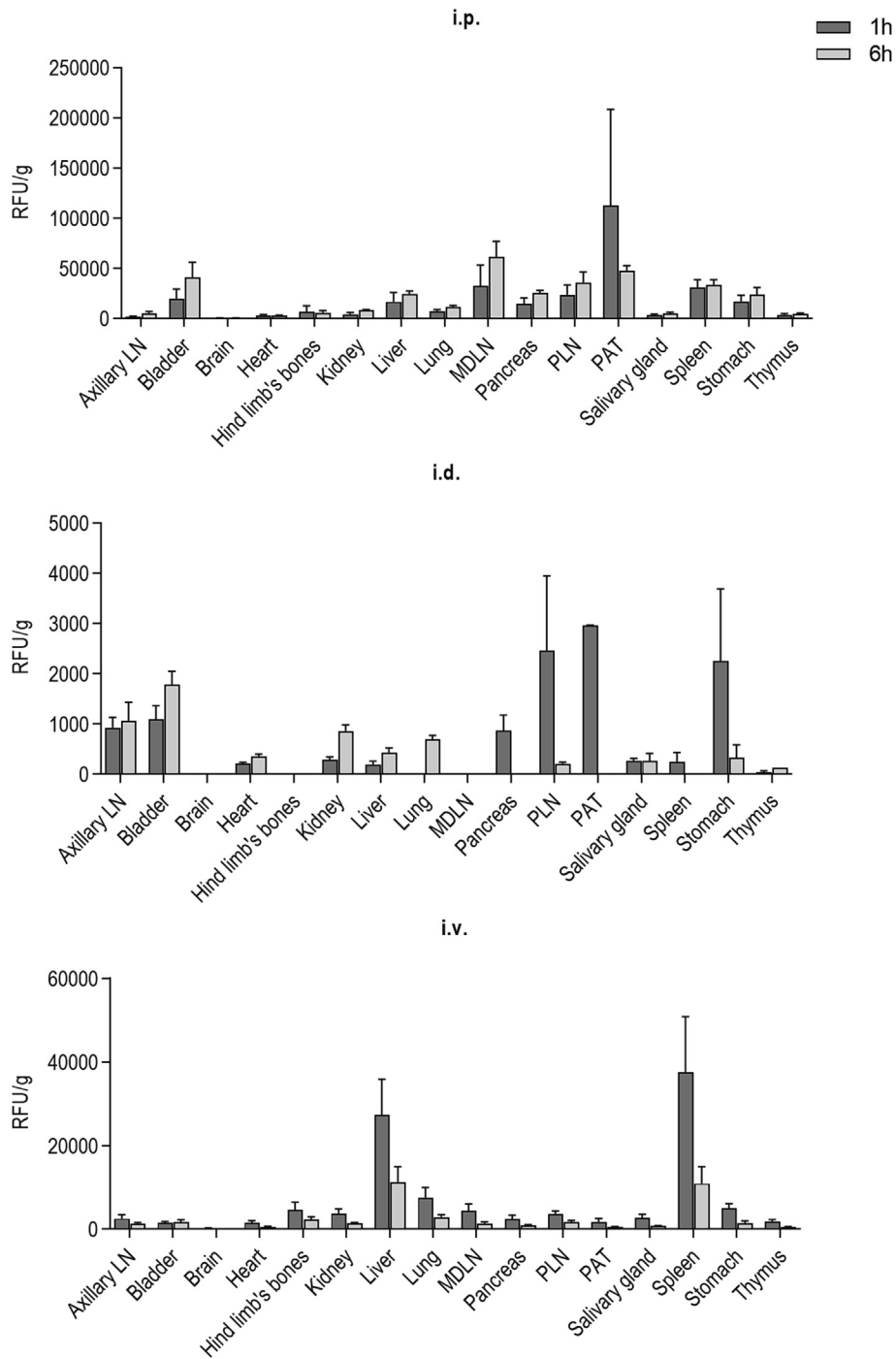


Fig. 1. Tracking of fluorescent DOPE-AF750-PS-liposomes. Histogram of relative fluorescent signal (RFU/g of tissue) in NOD mice 1 and 6 h after DOPE-AF750-PS-liposomes administration. Mice received 200 μ l of liposomes by intraperitoneal route (i.p., $n = 4$), 50 μ l of liposomes by intradermal route (i.d., $n = 3$) or 100 μ l by intravenous route (i.v., $n = 5$). No statistically differences were found between groups. Results are presented as mean \pm SEM.

liposomes, as a control, *via* i.v. and i.d. Their effect on T-cell subsets was determined in the spleen at 4- or 13-days post-treatment. Splenocytes viability was always higher than 82 %, discarding any toxicity due to empty or loaded PS2.5mi-liposomes (Fig. 4a, upper panel). Also, the number of total cells tended to be higher in mice treated with antigen-loaded PS2.5mi-liposomes (Fig. 4a, bottom panel). Mice treated i.d. showed a significantly higher number of splenocytes 4 days after PS2.5mi-

liposomes treatment when compared to 13 days post-treatment and to control mice. Despite non-significant, i.v. treatment showed the same trend.

Regarding the percentage of CD4⁺Foxp3⁺ T-cells, no differences were observed when comparing the treated and control group. However, total counts significantly increased after i.v. PS2.5mi-liposomes administration, decreasing at day 13 (Supplementary Fig. 3). Then, we analyzed the variations in the

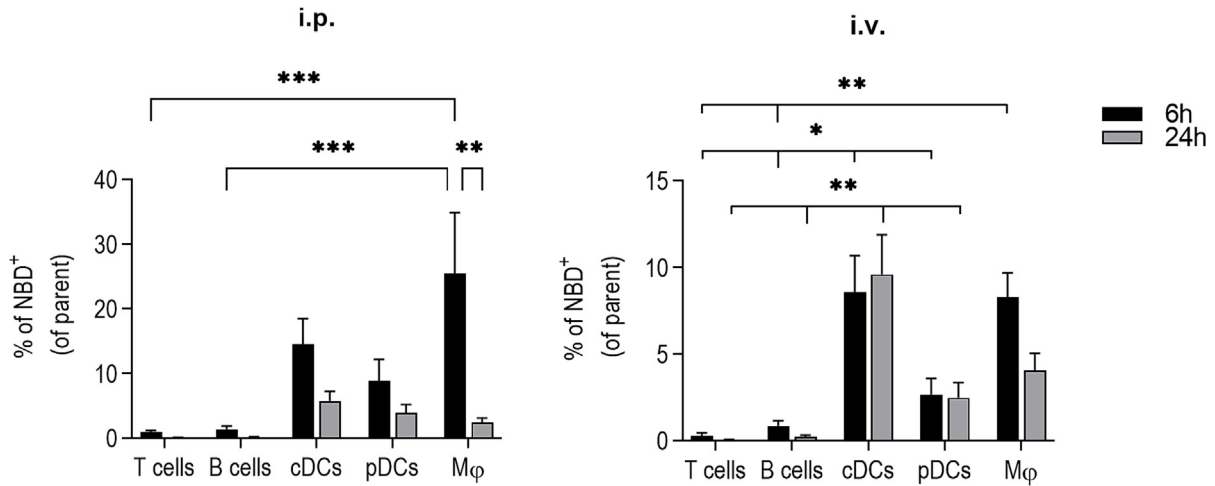


Fig. 2. Percentages of NBD-fluorescent cells of the parent gate in the spleen of animals treated with NBD-PSIns(h)-liposomes, i.p. or i.v., after 6 h ($n = 5-6$) or 24 h ($n = 5$) of treatment. Cells were determined as follows: T-cells, $CD3^+$; B-cells, $CD19^+$; conventional DCs (cDCs), $CD11c^+MHC-II^+CD205^+CD8a^+$; plasmacytoid DCs (pDCs), $CD11c^+MHC-II^+B220^+$; macrophages (Mφ), $F4/80^+$. Data are mean \pm SEM; differences were found when comparing immune subsets and timepoints within the same subset (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-way ANOVA with Tukey's multiple comparison test).

$CTLA4^+Foxp3^+ CD4^+$ T-cell subset (Fig. 4b, left panels). The percentage and number of $CTLA4^+Foxp3^+ CD4^+$ T-cells after PS2.5mi-liposomes treatment i.v. were higher than controls at day 4 post-injection. In the i.d. route, its percentage and number significantly decreased on day 13 after treatment. Regarding $CTLA4^+Foxp3^+ CD8^+$ T-cells, a significant increase in cell counts was found 4 days after administration of PS2.5mi-liposomes (both routes) in comparison to PS-liposomes, decreasing at day 13 (Fig. 4b, middle panels). The TR1 subset ($CD4^+Foxp3^-LAG3^+CD49b^+$) showed low percentages in all conditions. However, total counts significantly increased 4 days after i.v. administration of PS2.5mi-liposomes (Fig. 4b, right panels).

Despite B-cells do not express a specific 2.5mi receptor, we determined the PS2.5mi-liposomes effect on Breg-cell subsets (Fig. 4c). Although non-significant, we observed a trend towards a higher percentage and cell counts of $CD19^+Foxp3^+$ subset 4 days after i.d. PS2.5mi-liposomes injection, compared to mice

treated with empty PS-liposomes (Fig. 4c, left panels). Regarding the i.v. administration, this trend was observed only in cell counts and persisted on day 13 post-injection. A significant increase was found in the counts of the $CD19^+CD1d^+LAG3^+$ B-cell subset 4 days after i.d. PS2.5mi-liposomes treatment, when compared to PS-liposomes-treated mice (Fig. 4c, right panels). On day 13, the difference was lost and levels of both groups returned to levels of the empty PS-liposomes group. Despite differences were not statistically significant, mice administered with PS2.5mi-liposomes i.v. showed a trend towards a higher percentage and counts.

Due to the tolerogenic potential of anergic and exhausted T-cells, we studied whether the therapy induced changes in these subsets. Anergic $CD4^+$ T-cells, although non-significant, showed a trend to increase (percentage and number) 13 days after i.v. PS2.5mi-liposomes treatment in comparison to PS-liposomes (Fig. 5a). Similarly, anergic $CD8^+$ T-cells showed a trend to

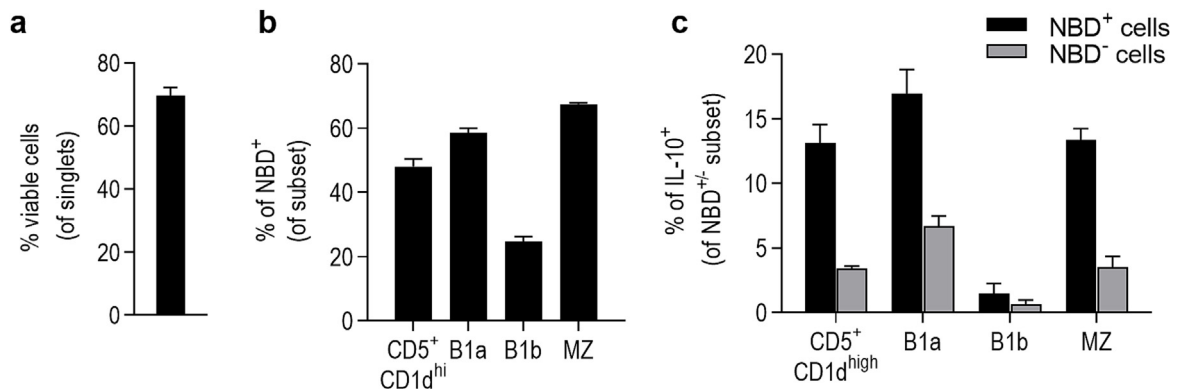


Fig. 3. Liposome-binding and IL-10 secreting B-cell subsets of culture splenocytes from NOD mice. a Viability in total NOD mice splenocytes after overnight cultured with NBD-PSIns(h)-liposomes ($n = 3$). b Percentage of NBD-fluorescent B-cell subsets within the whole subset gate after NBD-PSIns(h)-liposomes culture overnight ($n = 3$). Breg-cell subsets were determined as follows: $CD19^+CD1d^{high}CD5^+$; B1a, $CD19^+CD5^+CD43^+$; B1b, $CD19^+CD5^-CD43^+$; marginal zone (MZ), $CD19^+CD21^{high}CD23^-$. Data presented as mean \pm SEM. c Percentage of IL-10-secreting cells from NBD-PSIns(h)-liposome-binding NBD $^+$ B-cell subsets ($n = 3$) and from non-liposome-binding NBD $^-$ B-cell subsets ($n = 3$). Data presented as mean \pm SEM; no differences were found.

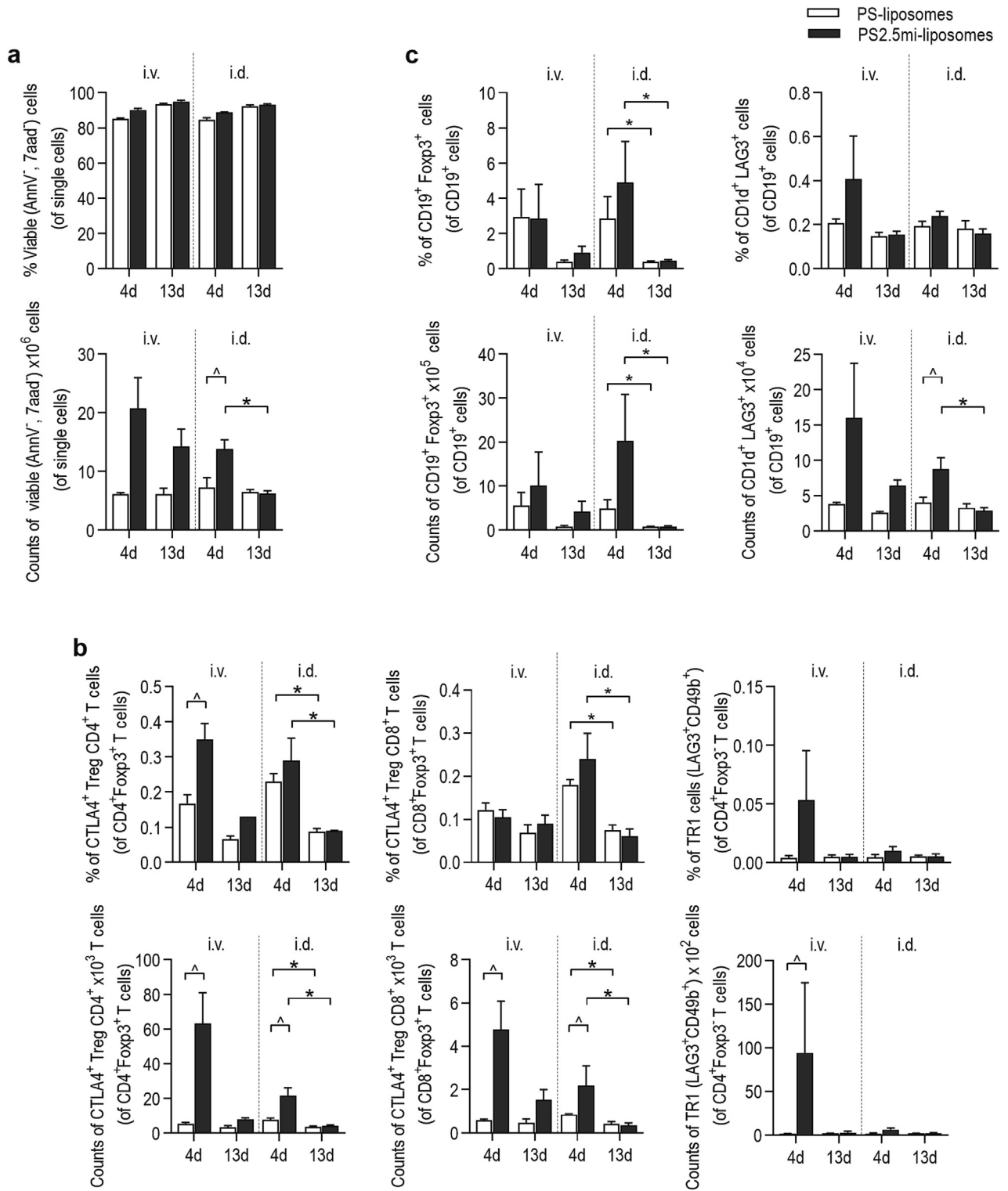


Fig. 4. Percentages and cell counts of (a) viable cells, (b) Breg-cell subsets – CD19⁺Foxp3⁺ and CD19⁺CD1d⁺LAG3⁺ B-cells– and (c) Treg-cell subsets – CD4⁺CTLA4⁺Foxp3⁺, CD8⁺CTLA4⁺Foxp3⁺ and TR1 CD4⁺Foxp3⁺LAG3⁺CD49b⁺ T-cells – from the spleen of NOD.BDC2.5 mice that received one dose of PS-liposomes or PS2.5mi-liposomes, *via* i.v. or i.d., and were euthanized after 4 days (n = 6) or 13 days (n = 6). Data are expressed as mean ± SEM, and significant differences were found when comparing the groups (^P < 0.05 between liposome treatment, *P < 0.05 between timepoints, Mann-Whitney test).

increase their counts after i.v. PS2.5mi-liposomes injection (Fig. 5b). Exhausted CD4⁺ T-cells showed higher counts 4 days after PS2.5mi-liposomes i.d. administration (and a tendency in i.v.

treatment) (Fig. 5c). Also, a significant increase in exhausted CD8⁺ T-cell counts was observed 4 days after i.v. treatment (Fig. 5d). Both exhausted T-cell subsets decreased 13 days after injection.

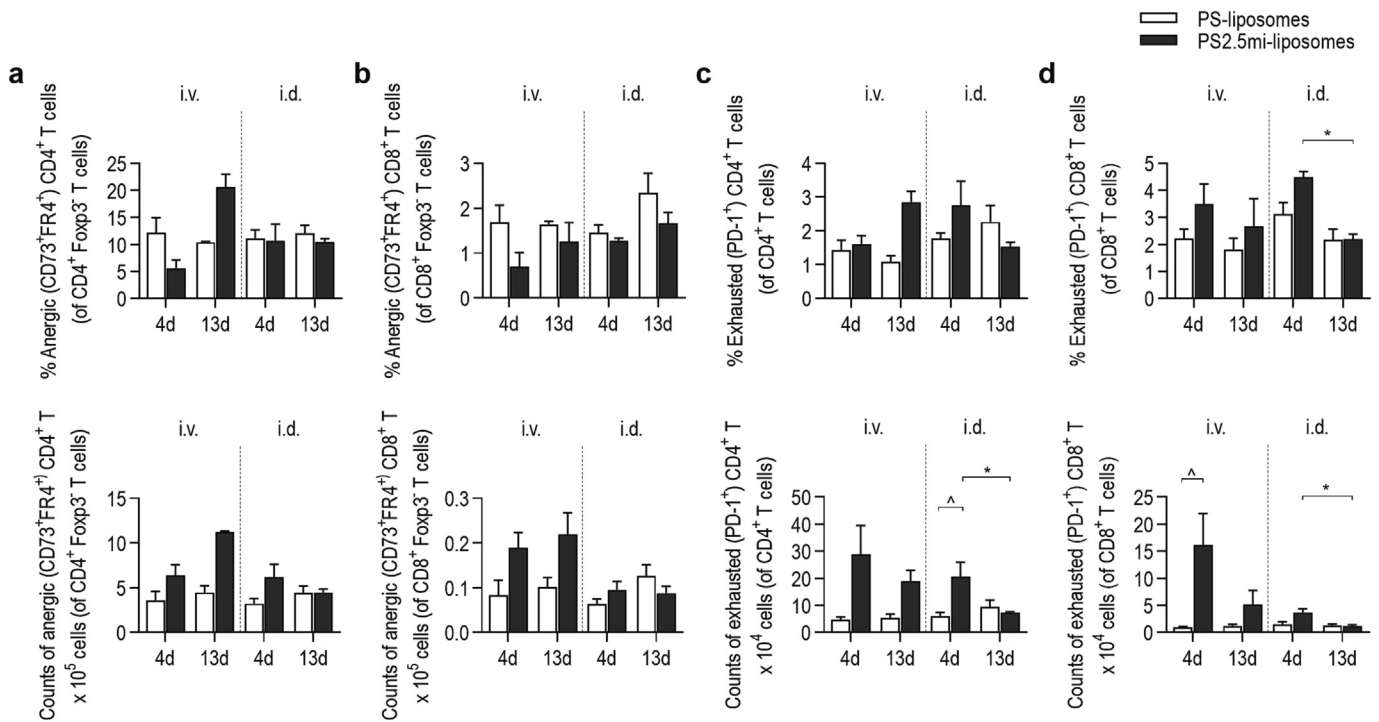


Fig. 5. Percentages and cell counts of (a) anergy $CD73^{+}FR4^{+} CD4^{+}$ T-cells, (b) anergy $CD73^{+}FR4^{+} CD8^{+}$ T-cells, (c) exhausted $PD-1^{+} CD4^{+}$ T-cells and (d) exhausted $PD-1^{+} CD8^{+}$ T-cells from the spleen of NOD.BDC2.5 mice that received one dose of PS-liposomes or PS2.5mi-liposomes, *via* i.v. or i.d., and euthanized after 4 days ($n = 6$) or 13 days ($n = 6$). Data are expressed as mean \pm SEM, and significant differences were found ($^{\wedge}P < 0.05$ between liposome treatment, $*P < 0.05$ between timepoints, Mann-Whitney test).

Antigen-specific PS-liposomes reduce rheumatoid arthritis severity

To broaden the spectrum of treatable AIDs with PS-liposomes, we tested antigen-specific PS-liposomes in experimental RA. Human RA is characterized by severe inflammation of peripheral joints in association with progressive degradation of articular cartilage and bone.¹² A well-established murine model for RA is AIA, which displays synovitis with infiltration of knee joints by neutrophils, macrophages, DCs and lymphocytes, pannus formation and subsequent cartilage and bone erosion.¹³ This model is achieved combining mBSA and MCV as an immunizing agent. Three days after disease induction, mice were treated *i.p.* with PSMCV-liposomes, PSVim-liposomes or PS-liposomes. As expected, immunized mice developed arthritis with notable knee joint swelling (Fig. 6a) whereas sham group remained healthy. A significant reduction in knee joint swelling was detected in PSMCV-liposomes-treated mice compared to the PS-liposomes group, while no change was found in PSVim-liposomes-treated mice (Fig. 6b). Flow cytometry analysis revealed a significantly increased number of $RANK^{+}CSFR^{+}$ osteoclast progenitor cells (OCPs) within the bone marrow of the empty PS-liposomes-treated immunized mice as compared to the PBS group (Fig. 6c). Bone-marrow resident OCPs were significantly decreased in PSMCV-liposomes and PSVim-liposomes-treated mice when compared to PS-liposomes-treated animals. Histological analysis revealed that PS-liposomes-treated mice exhibited notable synovitis and local bone erosion (Fig. 6d–f), whereas mice injected with PSMCV-liposomes confirmed an

amelioration of arthritis, showing a decrease in local bone erosion. Accordingly, PSMCV-liposomes-treatment caused a strong reduction of osteoclast numbers in the knee joints when compared to PS-liposomes treatment (Fig. 6g).

Myasthenia gravis specific PS-liposomes ameliorate disease severity and prevent the high production of IgG anti-AChR

Then, we developed liposomes for MG to determine their therapeutic power. MG is caused by autoantibodies directed against neuromuscular junction components such as the AChR¹⁴ leading to abnormal muscle fatigability. In the EAMG model, the disease was induced by immunization with AChR purified from *Torpedo californica* fish (T-AChR). Thus, at the moment of PS-liposomes administration, >80 % of immunized mice showed a GCS of 2 out of 9 on average (Fig. 7a). Control mice were non-immunized but injected with CFA, remaining healthy. Immunized mice were then treated *i.p.* with PS-liposomes, antigen-irrelevant PSmMOG-liposomes, or specific PSACHR-liposomes. Fig. 7b shows the GCS evolution, in which the PSACHR-liposomes group displays a GCS similar to the control CFA group. By contrast, mice treated with PS-liposomes or PSmMOG-liposomes showed a significantly higher GCS than the control group. This amelioration of clinical signs in the PSACHR-liposomes group was accompanied by a weight gain as the control CFA group (Fig. 7c).

Next, specific immunoglobulins against T-AChR were measured after PS-liposomes treatment on day 50 post-immunization. PSACHR-liposomes group had lower levels of anti-T-

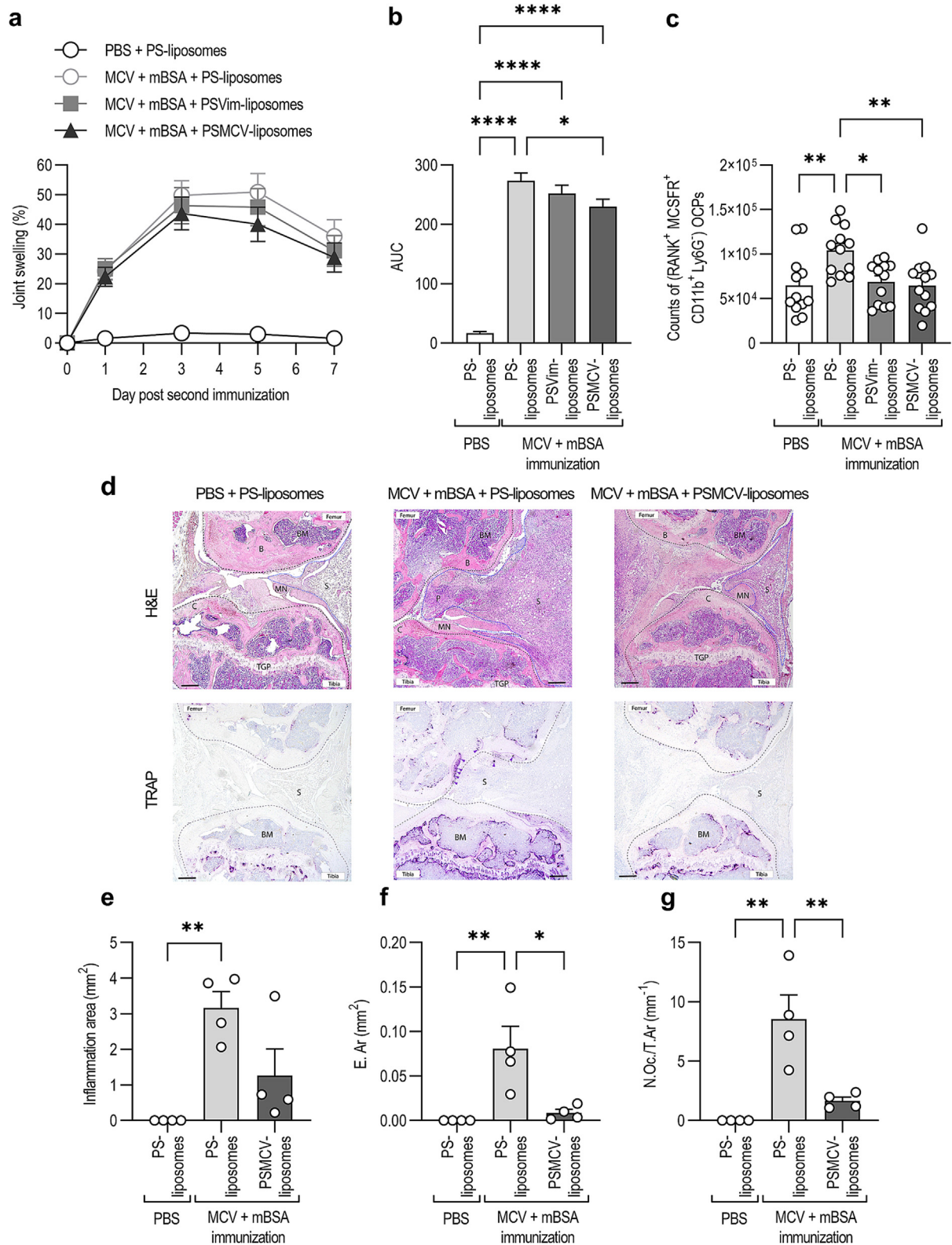


Fig. 6. Effect of PS-liposomes in experimental RA. a Knee swelling and (b) corresponding AUC measured in C57BL/6NRj mice immunized with a combination of MCV and mBSA or a PBS control, and treated with empty PS-liposomes or PSVim-liposomes or PSMCV-liposomes (n = 12). c Counts of OCPs (RANK⁺MCSFR⁺ CD11b⁺Ly6G⁻) from bone marrow 14 days after the first immunization. d Representative images of the histological analysis of H&E and TRAP stained sagittal sections through the knee joint; abbreviations are used as follows: cartilage (C), bone (B), bone marrow (BM), meniscus (MN), synovium (S), tibial growth plate (TGP); dashed lines border the tibia and the femur; blue dotted lines border the synovium in the H&E stained sections; purple dotted lines indicate the eroded surface; purple arrow tips indicate osteoclasts at the eroded area; scale bar equals 200 μ m. e Quantification of inflammation area, (f) eroded area (E.Ar), and (g) number of osteoclasts per tissue area (N.Oc./T.Ar). Data are expressed as mean \pm SEM, and significant differences were found between groups (*P < 0.05; **P < 0.01; ****P < 0.0001, One-way ANOVA test).

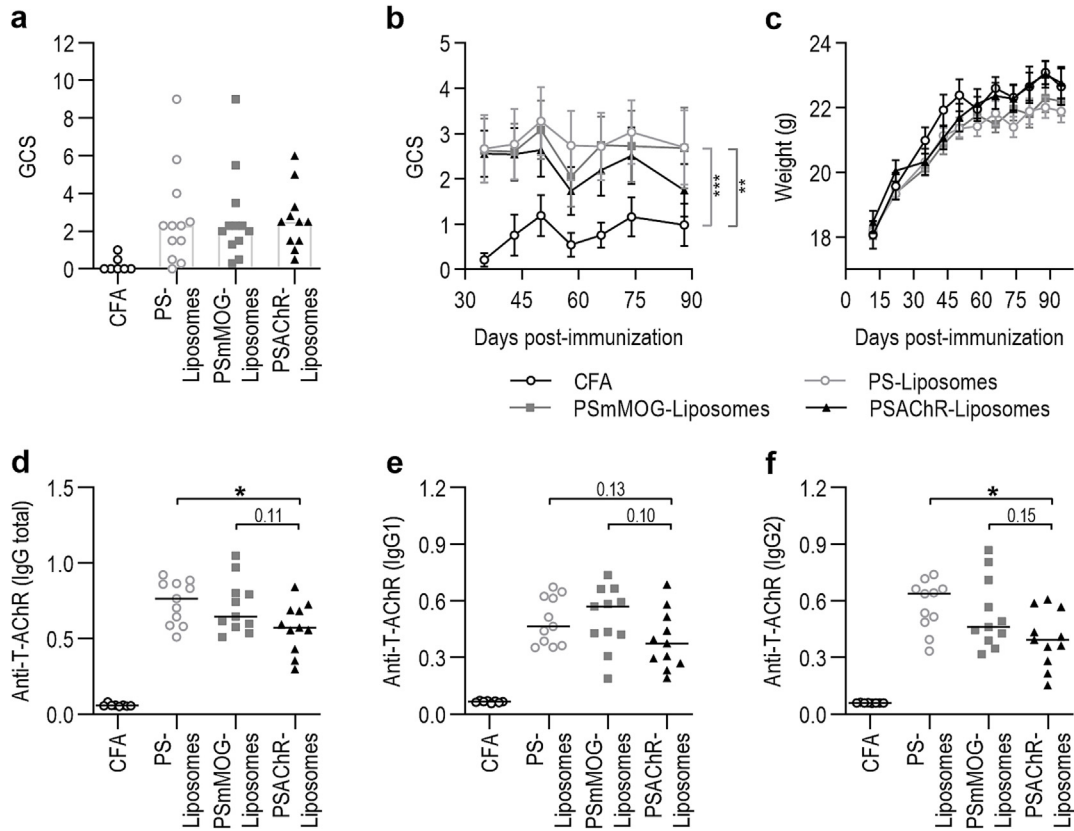


Fig. 7. Effect of PS-liposomes in experimental MG. a GCS of mice after two T-AChR immunizations and prior to empty PS-liposomes ($n = 12$), PSmMOG-liposomes ($n = 12$) or PSACHR-liposomes ($n = 11$) administration. Mice injected with CFA ($n = 7$) were non-immunized. b GCS evolution and (c) weight evolution in mice of the different groups. d Levels of anti-T-AChR total IgGs, (e) anti-T-AChR IgG1 and (f) anti-T-AChR IgG2 of the different groups at day 50 post-immunization. Data are presented as mean \pm SEM, and significant differences were found when comparing the groups ($*P < 0.05$; $**P < 0.01$; $****P < 0.0001$, One-way ANOVA test with Dunn's multiple comparison test).

AChR total IgGs in sera in comparison to the empty PS-liposomes group, and the same trend was observed when compared to the PSmMOG-liposomes group (Fig. 7d). This result was similar when IgG1 and IgG2b subtypes were analyzed separately (Fig. 7e, f). Finally, when analyzing anti-T-AChR total IgGs, IgG1 and IgG2 in sera throughout the study, PS-liposomes and PSmMOG-liposomes group had a significantly increased concentration in comparison to the CFA control, while the differences between the PSACHR-liposomes group and the CFA control group were not significant (Supplementary Fig. 4).

Discussion

An essential goal to prevent and cure AIDs is to re-establish self-tolerance, thus arresting the autoimmune reaction. A physiological process that promotes tolerance is efferocytosis, apoptotic cell engulfment. These dying cells exhibit surface PS to be distinguished from living cells, and to allow for their recognition by phagocytes and subsequent tolerance induction to self-antigens by antigen presentation.^{15,16} This process induces an immunologically silent microenvironment that maintains homeostasis and prompts tolerance to specific autoantigens, but also promotes growth, aiming at replacing cell loss.^{17,18}

Following these principles, a cell immunotherapy using apoptotic β -cells was demonstrated efficacious in re-establishing peripheral tolerance lost in T1D.¹⁹ Because autologous apoptotic cells cannot be easily obtained and standardized, lipid nanoparticles rich in PS and containing autoantigens were generated with a large diameter size to mimic apoptotic cells and harness their tolerogenic effect *via* their phagocytic engulfment by APCs.^{5,20} After internalization, PS and autoantigen enforce a binary signal for the APC to perform a tolerogenic antigen presentation, thus providing the basis for tolerance to self-proteins. Thus, these PS-liposomes encapsulating autoantigens constitute an innovative immunotherapy that halts autoimmunity in experimental T1D and MS,^{5,6} being a powerful tool for preventive and therapeutic use in AIDs.

An important issue for this immunotherapy is its biodistribution, which enables an effective local and systemic effect. The results suggest that the i.d. route displayed a rather localized effect in the vicinity of the injection site, whereas the i.p. and i.v. injection of PS-liposomes were better routes of administration than the i.d. in terms of rapid biodistribution, higher signal intensity and reaching target secondary lymphoid organs like the spleen. However, the i.p. route is not easily applicable to humans, and so the i.v. route appears more encouraging in terms

of translationality. As expected of a biodegradable and biocompatible liposomal platform²¹ the fluorescent signal decreased after 24 h of injection regardless of the route used, revealing little bioaccumulation. Previously, we have confirmed the fast *in vitro* capture of PS-liposomes by DCs, both murine and human.^{5–8} Here, we confirmed that DCs and macrophages engulfed PS-liposomes *in vivo* 6 h after injection. This result was expected due to their phagocytic function, and is very relevant to understand the therapeutic effect of the immunotherapy through tolerogenic antigen presentation. Rather unexpectedly, PS-liposomes interact with a small percentage of B lymphocytes, which play a critical role (pathogenic and regulatory) in T1D.^{22,23} Interestingly, this interaction was observed in B-cell subsets with regulatory potential and IL-10 production,²⁴ such as CD5⁺ CD1d^{hi}, B1a and MZ B-cells. In our hands, IL-10 secretion showed a biological trend to be higher in B-cells that interacted with PSIns(h)-liposomes when compared to non-liposome-binding B-cells. Since B-cells express PS-receptors and have reported regulatory potential after interacting with apoptotic cells,^{25,26} our data indicate that PS-liposomes have a tolerogenic effect in B-cells as well.

Interestingly, in NOD.BDC2.5 mice, *in vivo* treatment with PS2.5mi-liposomes increases the number of splenocytes only 4 days after administration in comparison to PS-liposomes, suggesting the expansion of autoantigen-specific cells. This specific treatment substantially increases the percentage and number of regulatory lymphocytic subsets, such as CTLA4⁺Foxp3⁺ CD4⁺ and CD8⁺ Treg-cells. Indeed, after phagocytosing PS2.5mi-liposomes, professional APCs like macrophages and DCs acquire tolerogenic features and promote Treg-cell formation and expansion,⁵ since upregulation of CTLA4 expression reportedly occurs on Foxp3⁺ Treg-cells undergoing extensive proliferation rates.²⁷ Nevertheless, due to the heterogeneous nature of Treg-cells, different subsets could be involved in various suppressive mechanisms. In fact, Treg-cells usually act through CTLA4 to enforce suppression, but CTLA4-independent mechanisms of suppression do exist. Therefore, Treg-cells expressing CD45RA produce more TGF- β , while the CD45RA-negative subset bears higher CTLA4 expression and greater IL-10 production.²⁸ Additionally, the number of TR1 cells increased after PS2.5mi-liposomes administration, reinforcing their tolerogenic effect. Indeed, other nanoparticles for AIDs induce TR1 cells as well.²⁹ Regarding Breg-cells, autoantigen-loaded PS-liposomes treatment expanded CD19⁺CD1d⁺LAG3⁺ cells and a rather unknown CD19⁺Foxp3⁺ subset, which is involved in decreasing severity in human RA³⁰ and its experimental model.³¹ On the other hand, tolDCs prompt anergic and exhausted T-cells, which could restrain autoimmunity. In fact, anergic T-cells are necessary to maintain homeostasis and arrest the progression to T1D in NOD mice,³² and autoreactive CD8⁺ T-cell exhaustion has been shown to distinguish subjects with slow T1D progression³³ and is associated with a beneficial response to therapy in recent-onset T1D.³⁴ Also, an exhausted T-cell phenotype inhibits epitope spreading in MG and reduces its severity in the experimental model.³⁵ Taken together, these results explain the regulatory effect of antigen-specific PS-liposomes induced by tolDCs and their tolerogenic antigen presentation after capture.⁵

Fitting well with our previous experience, we have demonstrated that RA and MG autoantigens can be efficiently encapsulated in PS-liposomes with optimal features for biomimicry and tolerance induction. These diseases were selected on the basis of previously identified autoantigens and different pathogenic mechanisms of disease. First, PSMCV-liposomes and PSACHR-liposome, for RA and MG treatment respectively, showed physicochemical features similar to those designed for T1D and MS.^{5,6} In both models, PS-liposomes were injected *i. p.* to compare their efficacy with the previously tested diseases.^{5,6} These specific liposomes significantly ameliorated the severity of both experimental diseases. In the RA model, apoptotic cell-mimicking PS-liposomes decreased synovitis and local bone erosion and prevented disease development. Our results are consistent with other authors that describe the beneficial effect of apoptotic cells in reducing joint inflammation,³⁶ and the role of tolDCs as therapeutic strategies for RA.³⁷ Moreover, autoantigen-loaded PS-liposomes induced IL-10 secretion in mouse Breg-cells and, in humans, Breg-cells have also been reported to inhibit RA progression,³⁸ reinforcing the potential of PS-liposomes. In the MG model, an amelioration of clinical symptoms was induced by PSACHR-liposomes. Because MG is an AID mainly caused by AChR autoantibodies,³⁹ the inhibition of specific immunoglobulins explains the effect of the liposome-based immunotherapy. Indeed, antibodies to AChR, specifically IgG2b isotypes, act *via* the complement pathway and are the most pathogenic autoantibodies, so the decrease in specific IgG2b agrees with the amelioration of disease severity. Thus, these changes confirm the effect of the PS-liposomes platform in specific humoral immunity as well as specific cellular immunity. In a future work, the therapeutic effect of PS-liposomes injected *i. v.* will be evaluated as well, to ensure clinical applicability in humans.

This work solidifies the ability of autoantigen-loaded PS-liposomes as a preventive and therapeutic platform in AIDs. The joint action of PS and autoantigen results in a successful effect in immune cells, which activate efferocytosis machinery and tolerogenic signal transduction. Indeed, the platform poses various advantages for future clinical use.⁴⁰

First, liposomes are reported as safe and biocompatible, biodegradable, non-immunogenic and non-toxic, since all components are already existing in mammals.²¹ In this sense, we previously evaluated welfare, behavioral and biochemical parameters in mice during and after severe PS-liposomes administration regimes, revealing a well-tolerated and safe profile.⁴¹ Also, PS-liposomes are easy to mass-produce, standardize and adapt to disease requirements by simply replacing the encapsulated autoantigen. Furthermore, their use has already been approved for a wide range of purposes,⁴² including SARS-CoV-2 mRNA vaccines.⁴³ In the field of AIDs, several liposome-based strategies are being developed as well. For example, liposomes without PS are being used to deliver anti-inflammatory drugs to control autoimmune inflammation in MS⁴⁴ or RA,⁴⁵ to deliver autoantigenic peptides for RA,⁴⁶ systemic inflammatory autoimmune diseases⁴⁷ or T1D,⁴⁸ and liposomes with PS and autoantigenic peptides are being trialed for experimental neuromyelitis optica.⁴⁹

Second, the platform is inspired by efferocytosis, a conserved physiological process that maintains self-tolerance, and is dependent on the presence of PS and large size to achieve successful tolerogenic induction.⁵⁰ We previously demonstrated that PS-lacking liposomes (PC-liposomes) do not expand putative regulatory antigen-specific CD4+ T cells as significantly as PS-liposomes do in NOD mice,⁵ and they are captured by human monocyte-derived DCs at a slower pace than PS-liposomes,⁷ evidencing that they do not resemble apoptotic cells as well as PS-rich liposomes. Thus, PS-liposomes interact with the immune system similarly to apoptotic cells, but with the advantage of being unable to degenerate into pro-inflammatory necrotic cells.

Third, the autoantigen promotes immune tolerance in an antigen-specific manner without compromising the rest of the immune response. Most importantly, the direct administration of PS-liposomes mimics the effect of autologous apoptotic cells and avoids *ex vivo* cell manipulation and invasive procedures for the patient, which is a clear advantage in comparison to cell-based therapies. Thus, PS-liposomes can generate a full-fledged tolerogenic immune response *in vivo* comprising tolDCs, regulatory macrophages, Treg-cells and Breg-cells.

We are well aware of the limitations of this work. Despite relevant data have been obtained from studies in experimental models with immunotherapies for AIDs,⁵¹ further work is required for translationality. Advancing in the preclinical phase pipeline, PS-liposomes immunomodulatory effect has been confirmed in human DCs from patients with T1D, which acquired global tolerogenic features after PS-liposomes phagocytosis in a similar way to mouse DCs.^{7,8} Work is in progress to confirm the effect of PS-liposomes in DCs from people living with MS, RA, MG and other AIDs.

In conclusion, we describe a simple method based on a powerful physiological process – efferocytosis – for inducing self-tolerance and for the prevention and treatment of different AIDs. Upon administration, PS-liposomes are distributed to target organs and captured by APCs, which mount a tolerogenic response involving classical and non-classical Treg-cells, Breg-cells, anergic and exhausted T lymphocytes, which in turn arrest the development of RA and MG. The PS-liposomes effect is owed to the combination of PS – tolerance signal – and autoantigens – the specificity signal – in a lipid vesicle, acting synergistically to re-educate the immune response and halt the autoimmune reaction. By simply replacing the encapsulated autoantigen, the PS-liposomes platform covers multiple antigen-specific AIDs, thus being a solid candidate strategy in the clinical setting.

Abbreviations

AIDs	Autoimmune diseases
APCs	Antigen-presenting cells
Breg-cell	Regulatory B-cell
MG	Myasthenia gravis
MS	Multiple sclerosis
NOD	Non-obese diabetic
PS	Phosphatidylserine

RA	Rheumatoid arthritis
T1D	Type 1 diabetes
tolDCs	Tolerogenic dendritic cells
Treg-cell	Regulatory T-cell

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Generalitat de Catalunya, Catalan Government. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Germans Trias i Pujol Research Institute (Permit DAAM 9521) and has followed the principles outlined in the Declaration of Helsinki for animal experimental investigation. Animal protocols for the RA model were approved by the Institutional Animal Care and by local ethics committee of Regierung von Mittelfranken, Germany. The protocol for the EAMG model was approved by the European and French ethics agreements (no. 23195).

CRediT authorship contribution statement

L.A.-F., S.R.-F., R.L.P., A.B., G.S., J.V., B.B.-Z., and M.V.-P. designed the experiments; B.B.-Z., S.R.-V., M.S., M.D., and M.V.-P. designed and manufactured the liposomes; L.A.-F., S.R.-F., E.R.-M., K.K., A.Y., and D.A. performed the experiments; L.A.-F., S.R.-F., E.R.-M., K.K., A.Y., and D.A. collected and analyzed data; U.S., H.B., A.B., G.S., R.L.P., J.V., and M.V.-P. provided expertise and materials for the establishment and follow-up of the murine models of disease; L.A.-F., S.R.-F., E.R.-M., K.K., R.L.P., and M.V.-P. wrote the manuscript; all authors revised the manuscript and gave final approval of the version to be published.

Declaration of competing interest

M.V.-P. is co-founder and CSO of Ahead Therapeutics SL. L.A.-F., S.R.-F., B.B. and S.R.-V., M.S., and M.D. are employees by this company. J.V. and M.V.-P. hold a patent that relate to liposome immunotherapy for autoimmune diseases, licensed to Ahead Therapeutics SL. This work was partially funded by Ahead Therapeutics SL. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

We are grateful to Mr. Marco Fernandez for his support in flow cytometry. This work was supported by Ahead Therapeutics SL, and the Agency for Management of University and Research Grants (AGAUR) of the Catalan Government (Fellowship to L.A.-F.). This work has been supported by positive discussion through Consolidated Research Group #2017 SGR 103, AGAUR, Generalitat de Catalunya.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nano.2022.102635>.

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