

**PRECISION TOLERANCE:
antigen-specific phosphatidylserine-liposome based platform
for autoimmune diseases**

Lidia Almenara Fuentes

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based platform for autoimmune diseases**

Lidia Almenara Fuentes

Doctoral Thesis

Badalona, 2025

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PhD programme in Advanced Immunology

Department of Cell Biology, Physiology and Immunology

Universitat Autònoma de Barcelona

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antigen-specific phosphatidylserine-liposome based platform for autoimmune diseases

Thesis presented by Lidia Almenara Fuentes to qualify for the PhD degree in Advanced Immunology with an industrial doctoral research component by the Universitat Autònoma de Barcelona.

The study presented was carried out jointly by the Immunology of Diabetes Unit of the Germans Trias i Pujol Research Institute (IGTP) and the company Ahead Therapeutics S.L., and directed by Dr Marta Vives Pi and Dr Bruna Barneda Zahonero.

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That the experimental work and the writing of the Doctoral Thesis entitled "Precision tolerance: antigen-specific phosphatidylserine-liposome based platform for autoimmune diseases" were conducted by Lidia Almenara Fuentes under her supervision and considers the dissertation suitable for presentation to opt for the degree of Doctor in Advanced Immunology.

And so for the record, she signs this document in Badalona,

Dr. Marta Vives Pi

A mi padre.

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ABBREVIATIONS

4-PL Four-parameter logistic regression model

5-PL Five-parameter logistic regression model

7-AAD 7-Amino-Actinomycin D

AC Apoptotic cells

AChR Acetylcholine receptor

ACK Ammonium-Chloride-Potassium

ACPAs Anti-citrullinated protein antibodies

ACR American College of Rheumatology

ADAMTS13 A disintegrin and metalloprotease with thrombospondin type 1 repeats 13

AF750 Alexa Fluor 750

AhR Aryl hydrocarbon receptor

AIRE Autoimmune regulator autoimmune regulator

ANNs Artificial Neural Networks

APCs Antigen-presenting cells

AUC Area under the curve

BAI1 Brain specific angiogenesis inhibitor 1

BCR B cell antigen receptor

Bregs B regulatory cells

BTLA B- and T-lymphocyte attenuator

CAR Chimeric antigen-specific receptor

CCL Chemokine ligand

CD Cluster of differentiation

cDCs Conventional dendritic cells

cDCs1 Conventional DCs type 1

cDCs2 Conventional DCs type 2

CH Cholesterol

CRS Cytokine release syndrome

CTLA-4 Cytotoxic T lymphocyte associated protein 4

CTV Cell trace violet

CXCL CXC motif chemokine ligand

CXCR CXC chemokine receptor

DAMPs Danger-associated molecular patterns

DAS28 Disease Activity Score 28

DCs Dendritic cells

DMARDs	Disease-modifying antirheumatic drugs
DMEM	Dulbecco's Modified Eagle Medium
DMPC	1,2-Dimyristoyl-sn-glycero-3-phosphocholine
DMSO	Dimethyl sulfoxide
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPS	1,2-dioleoyl-sn-glycero3-phospho-L-serine
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiencies
EMA	European medicines agency
ESR	Erythrocyte sedimentation rate
EULAR	European Alliance of Associations for Rheumatology
FDA	Food and drug administration
FITC	Fluorescein
FMO	Fluorescence minus one
FoxP3	transcription factor forkhead box P3
GAD65	Glutamic acid decarboxylase 65-kilodalton isoform
GH	Global health
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HSA	Human serum albumin
i.d.	Intradermal
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
IC50	Half-maximal inhibitory concentration
ICAM3	Intracellular adhesion molecule 3
ICOS	Inducible T cell co-stimulator
ICOSL	Inducible T cell co-stimulator ligand
iDCs	Immature dendritic cells
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
iNKTs	Invariant natural killer T cells

Ins	Insulin
ITE	2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester
ITTP	Immune thrombocytopenia purpura
JAK	Janus kinase
KHCO₃	Potassium bicarbonate
LAG3	Lymphocyte-activation gene 3
LC-MS/MS	Liquid chromatography–mass spectrometry
LN	Lymph nodes
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LRP4	Low-density lipoprotein receptor-related protein 4
LSECs	Liver sinusoidal endothelial cells
mABs	monoclonal antibodies
MARCO	Macrophage receptor with collagenous structure
M-CSF	Macrophage colony stimulating factor
MCV	Mutated citrullinated vimentin
mDCs	Mature dendritic cells
MDLN	Mediastinal lymph nodes
MG	Myasthenia gravis
MGFA	Myasthenia Gravis Foundation of America Classification
MHC	Major histocompatibility complex
moDCs	Monocyte-derived dendritic cells
MoMφ	Monocyte-derived macrophages
MPS	Mononuclear phagocyte system
MS	Multiple sclerosis
MS/MS	Tandem mass spectrometry
mTECs	Medullary thymic epithelial cells
MuSK	Muscle-specific kinase
Mφ	Macrophages
NaCl	Sodium chloride
NaHCO₃	Sodium bicarbonate
NBD	3-hexanoyl-Nitrobenzoxadiazole
NF-κB	Nuclear factor κB
NH₄Cl	Ammonium Chloride
NIR	Near-infrared
NKs	Natural killer cells
NMJ	Neuromuscular junctions

NMO	Neuromyelitis optica
NMR	Nuclear magnetic resonance-based
NOD	Non-obese diabetic
NP	Nanoparticle
PAT	Perigonadal adipose tissue
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PD1	Programmed cell death 1
pDCs	Plasmacytoid dendritic cells
PdI	Polydispersity index
PD-L1	Programmed cell death ligand 1
PEG	Pegylated
PGE₂	Prostaglandin E2
PLGA	Poly(lactic-co-glycolic) acid
PLN	Pancreatic lymph nodes
PRR	Pattern recognition receptors
PS	Phosphatidylserine
RA	Rheumatoid arthritis
RAG	Recombination-activating genes
RAGE	Receptor for advanced glycation end-products
RANKL	Receptor activator of nuclear factor κ B ligand
RBC	Red blood cells
RES	Reticuloendothelial system
RF	Rheumatoid factor
RFU	Relative fluorescence units
RPE	Streptavidin–R-phycoerythrin
RT	Room temperature
S1P	Lipids sphingosine 1-phosphate
scRNA-seq	Single-cell RNA sequencing
SF	Synovial fluid
SFDCs	Synovial fluid derived dendritic cells
SFMCs	Synovial fluid mononuclear cells
SJC	Swollen joint count
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
SPF	Specific-pathogen-free

T1D	Type 1 diabetes
TCR	T cell antigen receptor
TEC	Cortical thymic epithelial cells
TFG-β	Transforming growth factor β
Tfh	Follicular T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-1	T cell immunoglobulin mucin receptor 1
TIM-3	Mucin domain-containing protein 3
TIM-4	T cell immunoglobulin mucin receptor 4
TJC	Tender joint count
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TPE	Therapeutic plasma exchange
Tr1	Non-conventional IL-10 producing T regulatory cells
Tregs	T regulatory cells
VISTA	V-domain Ig suppressor of T cell activation
VNTRs	Variable numbers of tandem repeats
VWF	von Willebrand factor

SUMMARY:

The breakdown of the immune tolerance drives the development of autoimmune diseases, the prevalence of which is steadily rising and currently affects an estimated 5–10% of the global population. Conventional therapeutic strategies predominantly focus on symptomatic relief, replacement th2 erapies, or broad-spectrum immunosuppression. These approaches fail to address the underlying immunological dysregulation and are often associated with significant adverse effects. While recent advances in immunotherapy have yielded promising strategies to modulate autoimmune responses, a critical need remains for safe antigen-specific approaches capable of restoring long-lasting immune tolerance. This doctoral work explores an innovative immunotherapeutic platform based on the biomimicry of apoptotic cell clearance mechanism. Specifically, phosphatidylserine (PS)-enriched liposomes encapsulating disease-relevant autoantigens were engineered to induce antigen-specific immune tolerance and restore homeostasis in autoimmune settings. Previous studies have demonstrated the adaptability of this approach to different autoimmune responses contributing to the safe long-term re-establishment of tolerance in the T1D and MS murine models. Furthermore, human dendritic cells (DCs) derived from T1D patients exhibited a tolerogenic phenotype and functional modulation following uptake of insulin-loaded PS-liposomes.

The central hypothesis of this work is that PS-liposome-based immunotherapy can safely and effectively restore antigen-specific tolerance in several antigen-driven autoimmune disorders by modulating interacting immune cells toward a tolerogenic state. The main objective was to gain an in-depth understanding of how the immunotherapy works to design the best approach to ensure efficacy and safety in the future clinical trial. To this end, different autoantigens were successfully encapsulated within PS-liposomes. Biodistribution studies in murine models using various administration routes identified intravenous (IV) delivery as the most clinically relevant, owing to its rapid systemic distribution and targeted accumulation in immunologically significant organs such as the liver and spleen, where professional phagocytes efficiently internalized the liposomes. To demonstrate antigen-specific immune modulation, transgenic BDC2.5mi mice were treated with PS-liposomes encapsulating the 2.5mi peptide. After four days, there was a notable expansion of antigen-specific conventional and non-conventional regulatory T cells, as well as regulatory B cell subsets. By day thirteen, the therapy also induced antigen-specific exhausted and anergic T cells, suggesting its potential to promote long-term immune tolerance. To assess translational relevance, peripheral blood mononuclear cells

(PBMCs) from patients with Myasthenia Gravis (MG) and Rheumatoid Arthritis (RA) were used to differentiate monocytes into DCs. Additionally, putative monocytes from the synovial fluid were also isolated and differentiated. These human DCs effectively internalized PS-liposomes and, upon uptake, exhibited a tolerogenic phenotype characterized by upregulation of PS receptors, HLA-DR, chemokine receptors, and key tolerogenic markers such as DC-SIGN and PD-L1, alongside a downregulation of costimulatory molecules such as CD86 and CD54. Notably, PS-liposome uptake also led to antigen processing and presentation via MHC class II molecules. In line with this, tolerised DCs presented antigen-specific tolerogenic functionality impairing the autologous CD4⁺ and CD8⁺ T cell. Moreover, human B cells also demonstrated the ability to internalize fluorescently labelled PS-liposomes. These cells subsequently produced elevated levels of the anti-inflammatory cytokines IL-10 and TGF- β compared to non-interacting B cells, further supporting the tolerogenic potential of the platform. Finally, to address the safety of the IV administration into humans, hemocompatibility assays and cytokine release syndrome (CRS)-associated cytokine profiling were performed yielding favourable safety outcomes. Additionally, a robust and reproducible potency assay was developed to evaluate the biological activity of each PS-liposome batch, providing a critical quality control tool for translational development. Collectively, these findings support the clinical translation of PS-liposome platform, highlighting its capacity to induce antigen-specific tolerance, modulate immune responses in both murine and human systems, and maintain a favourable safety profile.

RESUMEN:

La pérdida de la tolerancia inmunológica es un factor clave en el desarrollo de las enfermedades autoinmunes, cuya prevalencia continúa en aumento y afecta actualmente al 5–10 % de la población mundial. Las estrategias terapéuticas convencionales se centran principalmente en el alivio sintomático, las terapias sustitutivas o la inmunosupresión de amplio espectro. Sin embargo, estos enfoques no abordan la disfunción inmunológica subyacente y con frecuencia se asocian a graves efectos adversos. A pesar de que los recientes avances en inmunoterapia han dado lugar a estrategias prometedoras capaces de modular las respuestas autoinmunitarias, persiste una necesidad crítica de desarrollar terapias seguras y específicas frente a autoantígenos capaces de restablecer una tolerancia inmunológica duradera. Este trabajo doctoral explora una plataforma inmunoterapéutica innovadora basada en la biomimética del mecanismo de eliminación de células apoptóticas. Concretamente, se diseñaron liposomas enriquecidos en fosfatidilserina (PS) que encapsulan autoantígenos relevantes para la enfermedad, con el objetivo de inducir tolerancia específica frente al antígeno y restablecer la homeostasis inmunológica. Estudios previos han demostrado la adaptabilidad de este enfoque a distintas respuestas autoinmunitarias, contribuyendo a la reinducción segura y duradera de la tolerancia en modelos murinos de diabetes tipo 1 (T1D) y esclerosis múltiple (EM). Además, células dendríticas (DCs) derivadas de pacientes con T1D mostraron un fenotipo tolerogénico y una modulación funcional tras la captación de liposomas de PS encapsulando insulina.

La hipótesis central de este trabajo es que la inmunoterapia basada en PS-liposomas puede restablecer de manera segura y eficaz la tolerancia específica frente a antígenos en diversas enfermedades autoinmunitarias, mediante la inducción de un perfil tolerogénico en las células inmunitarias que interactúan con ellos. El objetivo principal fue profundizar en la comprensión del mecanismo de acción de esta inmunoterapia, con el fin de diseñar el enfoque más adecuado que garantice su eficacia y seguridad en un futuro ensayo clínico. Para ello, se encapsularon con éxito diferentes autoantígenos en los PS-liposomas. Los estudios de biodistribución realizados en modelos murinos, mediante distintas vías de administración, identificaron la vía intravenosa (IV) como la más relevante desde el punto de vista clínico, debido a su rápida distribución sistémica y su acumulación específica en órganos inmunológicamente relevantes como el hígado y el bazo, donde fagocitos profesionales internalizaron eficazmente los PS-liposomas. Con el fin de demostrar

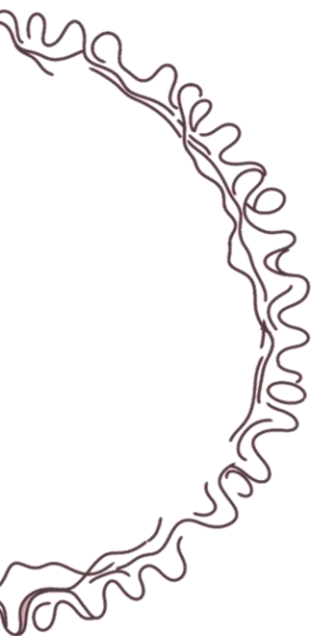
una modulación antígeno-específica, se trataron ratones transgénicos BDC2.5mi con PS-liposomas encapsulando el péptido 2.5mi. A los cuatro días de tratamiento, se observó una expansión significativa de células T reguladoras convencionales y no convencionales específicas del antígeno, así como de subconjuntos de células B reguladoras. Al decimotercer día, la terapia también indujo células T específicas del antígeno con un fenotipo exhausto y anérgico, lo que indica un potencial para promover una tolerancia inmunológica a largo plazo. Para evaluar la relevancia traslacional, se utilizaron células mononucleares de sangre periférica (PBMCs) de pacientes con Miastenia Gravis (MG) y Artritis Reumatoide (AR) para diferenciar monocitos en células dendríticas. Asimismo, se aislaron y diferenciaron presuntos monocitos del líquido sinovial. Estas DCs humanas internalizaron eficazmente los PS-liposomas y, tras su captación, adquirieron un fenotipo tolerogénico caracterizado por la sobreexpresión de receptores de PS, HLA-DR, receptores de quimiocinas y marcadores tolerogénicos clave como DC-SIGN y PD-L1, junto con una disminución de moléculas coestimuladoras como CD86 y CD54. Así mismo, la captación de liposomas y su procesamiento condujo a la presentación de antígeno encapsulado a través de moléculas del MHC de clase II. En coherencia con ello, las DCs tolerizadas mostraron una funcionalidad tolerogénica específica del antígeno, inhibiendo la proliferación autóloga de linfocitos T CD4⁺ y CD8⁺. Por otro lado, células B humanas también demostraron la capacidad de internalizar PS-liposomas marcados fluorescentemente. Tras la captación, estas células produjeron mayores niveles de las citoquinas antiinflamatorias IL-10 y TGF- β en comparación con las células B incapaces de interactuar. Finalmente, para abordar la seguridad de la administración IV en humanos, se realizaron ensayos de hemocompatibilidad y análisis de citoquinas asociadas al síndrome de liberación de citoquinas (CRS), obteniéndose resultados favorables. Además, se desarrolló un ensayo de potencia robusto y reproducible capaz de evaluar la actividad biológica de cada lote de PS-liposomas, proporcionando una herramienta esencial de control de calidad para el desarrollo traslacional. En conjunto, estos resultados respaldan la viabilidad clínica de la plataforma, destacando su capacidad para inducir tolerancia específica frente a antígenos, modular respuestas inmunitarias tanto en modelos murinos como en humanos, y mantener un perfil de seguridad favorable.

RESUM:

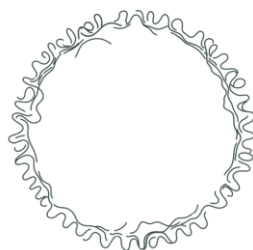
La pèrdua de la tolerància immunològica és un factor clau en el desenvolupament de les malalties autoimmunes, la prevalença de les quals continua en augment i afecta actualment al 5–10 % de la població mundial. Les estratègies terapèutiques convencionals se centren principalment en l'alleujament simptomàtic, les teràpies substitutives o la immunosupressió d'ampli espectre. No obstant això, aquests enfocaments no aborden la disfunció immunològica subjacent i amb freqüència s'associen a greus efectes adversos. A pesar que els recents avanços en immunoteràpia han donat lloc a estratègies prometedores capaces de modular les respostes autoimmunitàries, persisteix una necessitat crítica de desenvolupar teràpies segures i específiques enfront d'autoantígens capaços de restablir una tolerància immunològica duradora. Aquest treball doctoral explora una plataforma immunoterapèutica innovadora basada en la biomimètica del mecanisme d'eliminació de cèl·lules apoptòtiques. Concretament, es van dissenyar liposomes enriquits en fosfatidilserina (PS) que encapsulen autoantígens rellevants per a la malaltia, amb l'objectiu d'induir tolerància específica enfront de l'antigen i restablir l'homeòstasi immunològica. Estudis previs han demostrat l'adaptabilitat d'aquest enfocament a diferents respostes autoimmunitàries, contribuint a la reinducció segura i duradora de la tolerància en models murins de diabetis tipus 1 (T1D) i esclerosi múltiple (EM). A més, cèl·lules dendrítiques (DCs) derivades de pacients amb T1D van mostrar un fenotip tolerogènic i una modulació funcional després de la captació de liposomes de PS encapsulant insulina.

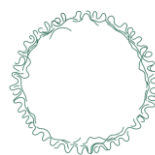
La hipòtesi central d'aquest treball és que la immunoteràpia basada en PS-liposomes pot restablir de manera segura i eficaç la tolerància específica enfront d'antígens en diverses malalties autoimmunitàries, mitjançant la inducció d'un perfil tolerogènic en les cèl·lules immunitàries que interactuen amb ells. L'objectiu principal va ser aprofundir en la comprensió del mecanisme d'acció d'aquesta immunoteràpia, amb la finalitat de dissenyar l'enfocament més adequat que garanteixi la seva eficàcia i seguretat en un futur assaig clínic. Per a això, es van encapsular amb èxit diferents autoantígens en els PS-liposomes. Els estudis de biodistribució realitzats en models murins, mitjançant diferents vies d'administració, van identificar la via intravenosa (IV) com la més rellevant des del punt de vista clínic, a causa de la seva ràpida distribució sistèmica i la seva acumulació específica en òrgans immunològicament rellevants com el fetge i la melsa, on fagòcits professionals van internalitzar eficaçment els PS-liposomes. Amb la finalitat de demostrar una modulació antigen-específica, es van tractar ratolins transgènics BDC2.5mi amb PS-liposomes

encapsulant el pèptid 2.5mi. Als quatre dies de tractament, es va observar una expansió significativa de cèl·lules T reguladores convencionals i no convencionals específiques de l'antigen, així com de subconjunts de cèl·lules B reguladores. Al tretzè dia, la teràpia també va induir cèl·lules T específiques de l'antigen amb un fenotip exhaust i anèrgic, la qual cosa indica un potencial per a promoure una tolerància immunològica a llarg termini. Per a avaluar la rellevància traslacional, es van utilitzar cèl·lules mononuclears de sang perifèrica (PBMCs) de pacients amb Miastènia greu (MG) i Artritis Reumatoide (AR) per a diferenciar monòcits en cèl·lules dendrítiques. Així mateix, es van aïllar i van diferenciar presumptes monòcits del líquid sinovial. Aquestes DCs humanes van internalitzar eficaçment els PS-liposomes i, després de la seva captació, van adquirir un fenotip tolerogènic caracteritzat per la sobreexpressió de receptors de PS, HLA-DR, receptors de quimiocines i marcadors tolerogènics clau com a DC-SIGN i PD-L1, juntament amb una disminució de molècules coestimuladores com CD86 i CD54. Així mateix, la captació de liposomes i el seu processament va conduir a la presentació d'antigen encapsulat a través de molècules del MHC de classe II. En coherència amb això, les DCs toleritzades van mostrar una funcionalitat tolerogènica específica de l'antigen, inhibint la proliferació autòloga de limfòcits T CD4⁺ i CD8⁺. D'altra banda, cèl·lules B humanes també van demostrar la capacitat d'internalitzar PS-liposomes marcats fluorescentement. Després de la captació, aquestes cèl·lules van produir majors nivells de les citocines antiinflamatòries IL-10 i TGF- β en comparació amb les cèl·lules B incapaces d'interactuar. Finalment, per a abordar la seguretat de l'administració IV en humans, es van realitzar assajos de hemocompatibilitat i anàlisis de citocines associades a la síndrome d'alliberament de citocines (CRS), obtenint-se resultats favorables. A més, es va desenvolupar un assaig de potència robust i reproduïble capaç d'avaluar l'activitat biològica de cada lot de PS-liposomes, proporcionant una eina essencial de control de qualitat per al desenvolupament traslacional. En conjunt, aquests resultats recolzen la viabilitat clínica de la plataforma, destacant la seva capacitat per a induir tolerància específica enfront d'antígens, modular respostes immunitàries tant en models murins com en humans, i mantenir un perfil de seguretat favorable



1





INTRODUCTION

1. The balance between immunity and autoimmunity

The immune system is a complex network of cells, tissues, and organs that coordinate to protect the body from a wide range of pathogens, potentially harmful agents and the spontaneous formation of neoplasms. It is broadly divided into two key components: the innate immune system, which provides a rapid, non-specific defence against pathogens, and the adaptive immune system, which responds with high specificity by B and T lymphocytes targeting specific pathogens and generating immunological memory. One of the most important features of the immune system is its ability to distinguish between self and non-self-antigens, a phenomenon known as immunological tolerance [1]. This tolerance against self-antigens is promoted by the processes involving the elimination, the inactivation and/or the suppression of self-reactive lymphocytes. The importance of immune tolerance cannot be overstated, as its failure may result in the immune system targeting the body's own cells and tissues, which would lead to a wide range of autoimmune conditions. Conversely, dysregulation of immune responses to harmless foreign antigens can contribute to hypersensitivity reactions and allergic disease. Understanding the complexities of immune tolerance is crucial for developing therapeutic strategies to modulate immune responses in multiple pathologies, including autoimmunity, allergy, and transplant rejection.

1.1. Mechanisms of tolerance

The breakdown of self-tolerance is a critical factor in the onset of autoimmune diseases. Therefore, understanding the multiple mechanisms involved in the establishment and maintenance of immune tolerance is essential. **Immune tolerance** refers to the long-term or permanent suppression of potentially harmful immune responses against specific, innocuous self-antigens. Every day, billions of lymphocytes are generated in the bone marrow and in the thymus through V(D)J recombination, producing a diverse repertoire of receptors capable of recognizing a wide range of antigens, including potential pathogens. However, this random process inevitably leads to the generation of some lymphocytes with receptors that recognize self-antigens. To prevent autoimmune responses, the immune system employs both central and peripheral mechanisms to regulate these self-reactive lymphocytes. Central tolerance mechanisms, occurring during lymphocyte development in the thymus or bone marrow, ensure the deletion or functional alteration of lymphocytes with high-affinity receptors for self-antigens. On the other hand, peripheral tolerance mechanisms further safeguard against autoreactivity by eliminating, inactivating, or

converting self-reactive lymphocytes into regulatory cells after they enter circulation. Collectively, these processes are fundamental to maintaining immune homeostasis and preventing the immune system from erroneously targeting the body's own tissues.

1.1.1. Central tolerance

Central tolerance is established during the development of immune cells, particularly T cells and B cells, in central lymphoid organs and has as an objective to shape the lymphocyte repertoire to limit the survival of self-reactive cells. Central tolerance of T lymphocytes occurs in the **thymus**, where bone marrow-derived CD34⁺ T cell progenitors migrate to commit and develop into CD4⁺ CD8⁺ double-positive thymocytes expressing the T cell antigen receptor (TCR). In the thymic cortex, double positive thymocytes undergo positive selection, where only survive those that have TCR binding to self-peptide:self-MHC complexes presented by cortical thymic epithelial cells (TEC), whereas those that fail to interact, undergo apoptosis. This process results in MHC restrictions, which triggers their commitment to either the CD4⁺ or CD8⁺ lineage, guiding their maturation into single-positive T lymphocytes and triggering their migration to the thymic medulla. There, the fate of developing T cells is determined through a process called negative selection, which is based on the affinity of their TCRs for self-antigen:self-MHC complexes. Antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages (Mφ), and B cells, contribute to this selection process. However, the primary regulators of this process are medullary thymic epithelial cells (mTECs), which express specialised transcription factors like autoimmune regulator (AIRE) and FEZF2 [2,3]. These transcription factors enable mTECs to adopt lineage-defining characteristics of peripheral cells, allowing them to present a diverse range of ectopic peripheral tissue-restricted antigens that otherwise would not be presented in the thymus [4]. Additional mechanisms such as the cooperative transfer antigen between mTECs and DCs enhance these cells to present tissue-specific antigens [5]. Interestingly, intestinal and peripheral DCs have been observed migrating to the thymus to present microbiota-derived and peripheral antigens, underscoring the contribution of peripheral DCs to central tolerance [6,7]. Additionally, a pathway has been identified where transendothelial DCs extend their dendrites across the endothelial barrier into the bloodstream, capturing circulating antigens and presenting them in the thymus [8]. Therefore, thymocytes carrying a TCR with high affinity for self-antigen:self-MHC molecules presented are deleted through apoptosis. Thymocytes with intermediate affinity are induced to differentiate into T regulatory cells (Tregs) expressing the

transcription factor forkhead box P3 (FoxP3) and finally, those with low affinity become conventional T cells, which are then released into the periphery.

In the **bone marrow**, the regulatory mechanisms that control B cell tolerance occur at the early stages of B cell development, when B cells carry a surface antigen receptor of the IgM class but are not fully mature. At this developmental stage, pre-B cells acquire the expression of a B cell antigen receptor (BCR), which is generated through the random rearrangement of their V, D, and J gene segments, leading to the formation of a diverse BCR repertoire [9]. However, a significant proportion—up to 75%— of early immature B cells express receptors that are autoreactive to self-antigens present on the surface of stromal cells [10]. To ensure self-tolerance and prevent autoimmunity, molecular quality control mechanisms are in place to eliminate B cells that fail to express a functional BCR or that exhibit autoreactivity. One-third of these self-reactive B cells undergo a process known as receptor editing, which involves the downregulation of BCR expression and the reactivation of recombination-activating genes (RAG). If receptor editing is unsuccessful or the autoreactive heavy chain (H chain) is not corrected through pairing with a non-autoreactive light chain (L chain), these B cells are eliminated via apoptosis. Following this process, B cells will then enter the spleen, lymph nodes, or other tissues.

Central tolerance eliminates or neutralizes self-reactive lymphocytes during their development in the thymus and bone marrow. However, this process is not fully infallible, and some self-reactive lymphocytes, particularly those targeting antigens not expressed in these organs, can escape. These autoreactive T and B cells then migrate to peripheral tissues, where peripheral tolerance mechanisms will regulate the unwanted immune activation of the autoreactive lymphocytes.

1.1.2. Peripheral tolerance

A significant percentage of self-reactive T cells and autoreactive B cells escape central tolerance [10,11]. Thus, peripheral tolerance mechanisms, including anergy, deletion and suppression by Treg cells, are crucial for the prevention of unwanted peripheral immune activation.

For T cell activation, are required three different signals. The first signal is the interaction between the TCR with the peptide:MHC complex. The second signal involves the binding of co-stimulatory receptors on the naïve T cell with their corresponding ligands on the APCs. The third signal is provided by cytokines, which activate cytokine receptors on the T cell. When the activation of the TCR occurs in the absence of co-stimulation, or a strong exposure to cytokines before the two first

signals happen, **T cell anergy** is induced [12]. This state can also be induced by repeated antigen stimulation [13], exposure to anti-inflammatory cytokines such as interleukin (IL)-10 [14], or signalling via co-inhibitory receptors such as programmed cell death 1 (PD1) and cytotoxic T lymphocyte associated protein 4 (CTLA4) [15]. In this state, T cell becomes functionally inactivate, unable to proliferate or produce functional cytokines even restimulation under robust stimulatory conditions. However, this refractory state is not permanent, and *in vivo* studies has shown that anergic T cells can gradually regain functional responsiveness in the absence of antigen. This suggests that the maintenance of T cell anergy is dependent on sustained antigen exposure, and without continuous interaction with the antigen, the anergic state can be reversed over time [16]. Interestingly, in line with the reversibility of anergic T cells, it has been shown that sometimes upon restimulation with the specific antigen can reverse their phenotype into FoxP3⁺ T cells or Tr1 cell [17]. This mechanism differs from quiescence and ignorance. **Quiescence** refers to a state in which T cells are alive but maintain a lower basal metabolic rate compared to their thymocyte counterparts, and this state occurs independently of antigen-TCR interaction. This process is regulated by specific quiescence mediators that maintain T cells in the G0 phase of the cell cycle, sustain a small cell size, limit cellular metabolism and prevent the development of effector functions in response to tonic signals, such self-antigens. The signalling pathways that control this stage are dynamic and respond to external cues, modulating the quiescence threshold in different environmental contexts controlling the responsiveness of naïve T cells [18]. In contrast, **ignorance** arises when T cells fail to recognize an antigen, either due to a low affinity for the antigen or a low antigen density and/or the restriction of antigen recognition owing to its anatomical location in immune privileged sites [19].

T cell deletion via apoptosis is an efficient and important mechanism for regulating the peripheral T cell repertoire and ensuring immune homeostasis by permanently removing T cell clones [20]. It serves not only to eliminate self-reactive T cells that escape central tolerance but also to terminate the immune response following successful pathogen clearance. A deficient co-stimulation of the TCR is also linked to T cell apoptosis by upregulating death receptors, however, the precise conditions determining whether a T cell undergoes anergy or apoptosis remain unclear. Antigen levels are though to influence cell fate, with higher levels triggering anergy and lower levels promoting cell death [21]. Other forms of immune tolerance-related cell death such as necroptosis, pyroptosis and ferroptosis, have been identified, regulated by different signalling pathways, yet all contributing to restrain T cell expansion at various stages of the immune response [22–24].

Although not typically classified as a peripheral tolerance mechanism, **T cell exhaustion** represents a key non-deletional tolerance mechanism in specific immunological situations. During acute infections or antigen recognition, effector T cells transition into either effector memory T cells or central memory T cells. Nevertheless, in cases of chronic or persistent antigen stimulation, the proper development of memory T cells can fail, leading to the development of exhausted T cells [25]. These cells are functionally impaired, characterised by a reduced antigen responsiveness, decrease in cytokine production, altered epigenetic, metabolic and transcriptional profiles, and elevated expression of inhibitory receptors including PD-1, CTLA4, Lymphocyte-activation gene 3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), Cluster of differentiation-(CD)-38, CD39, and T cell immunoglobulin (Ig) domain and mucin domain-containing protein 3 (TIM3) [26]. This exhausted phenotype renders T cells more resistant to activation and therefore less aggressive. This outcome has been proposed to be a protective mechanism designed to protect the host during periods of prolonged activation through prevention of persistent inflammation, tissue damage, and chronic auto-reactivity, being essential for long-term host survival [27,28].

Another important mechanism of regulation is mediated by **T regulatory cells** (Tregs), which suppress the activity autoreactive T cells that escaped central deletion as well as T cells that cross-react with self-antigens due to molecular mimicry with microbial antigens [29]. Key Treg cell subsets include conventional FoxP3⁺ Tregs, non-conventional IL-10 producing Tr1 cells, and additionally subsets such as CD8⁺ Treg cells, invariant natural killer T cells (iNKTs) and regulatory $\gamma\delta$ T cells [30–32]. Some Tregs are thymus-derived that already express FoxP3 during their development, while others differentiate from CD4⁺ naïve T cells in the periphery. This differentiation occurs following the TCR-antigen recognition without additional co-stimulation or the presence of anti-inflammatory cytokines such as IL-10 or transforming growth factor (TFG)- β anti-inflammatory cytokines. Similarly, Tr1 cells are generated through chronic TCR activation in the presence of IL-10 [33]. The immunosuppressive functions of Tregs relies on multiple mechanisms, including the direct or indirect inhibition of T effector cell activity, production of anti-inflammatory cytokines, and the generation of additional Treg cells or regulatory APCs, thereby skewing immune activity towards tolerance [34,35].

Self-reactive B lymphocytes are silenced in the periphery when their B cell receptor (BCR) binds to an antigen with high avidity in the absence of toll-like receptors (TLR) signals or T-helper cell co-stimulation, leading to clonal deletion or anergy, which prevents B cell proliferation and antibody production [36]. However, while B cells are

traditionally recognized for their capacity to differentiate into antibody-producing plasma cells, they also have other important functions, such as antigen presentation and the secretion of a wide range of pro- and anti-inflammatory cytokines. A specialized subset, known as **regulatory B cells** (Bregs), is responsible for producing immunosuppressive cytokines such as IL-10, TGF- β and IL-35 (**Figure 1**). Their differentiation is influenced by specific metabolic stimuli during BCR engagement and soluble microenvironmental signals, shaping the B cell compartment to promote immune regulation [37,38]. These Bregs play a key role in maintaining tolerance, limiting ongoing immune responses by skewing naïve T cells differentiation into Tregs and Tr1, suppressing pro-inflammatory cytokine production by innate immune cells, promoting apoptosis of CD4⁺ T cells and anergy of CD8⁺ T cells, and maintaining iNKTs cell homeostasis [39–43].

The activation and maturation states of **DCs** also play a crucial role in shaping the immune response and in self-tolerance. This maturation stage is triggered by the activation of the pattern recognition receptors (PRR) and involves increased expression of MHC class I and II molecules, co-stimulatory molecules and adhesion molecules. Then, mature DCs migrate to the lymph nodes where they will drive the differentiation of effector T cells. However, depending on how the antigen is perceived during the uptake, DCs can be matured in the absence of these stimulus and present self-antigens to autoreactive T cells that escaped thymic selection [44]. This process, known as tolerogenic or homeostatic maturation, can be induced by different stimuli that activates specific transcriptional programmes, resulting in the differentiation of tolerogenic DCs (for more detailed information, see **section 3.1.2**). These DCs contribute to the establishment of peripheral tolerance through several mechanisms, including the downregulation of co-stimulatory molecules (CD80, CD86 and CD40), upregulation of inhibitory molecules (programmed cell death ligand 1 (PD-L1), inducible T cell co-stimulator ligand (ICOSL) and B- and T-lymphocyte attenuator (BTLA), suppression of proinflammatory cytokines (IL-6, IL-12, IL-23 and tumour necrosis factor (TNF)) and the production of anti-inflammatory cytokines (IL-10, TGF- β and IL-27) and metabolites (indoleamine-pyrrole 2,3-dioxygenase (IDO), retinoic acid and lactate). Collectively, these actions promote T cell anergy, deletion, or the generation of regulatory lymphocytes [45].

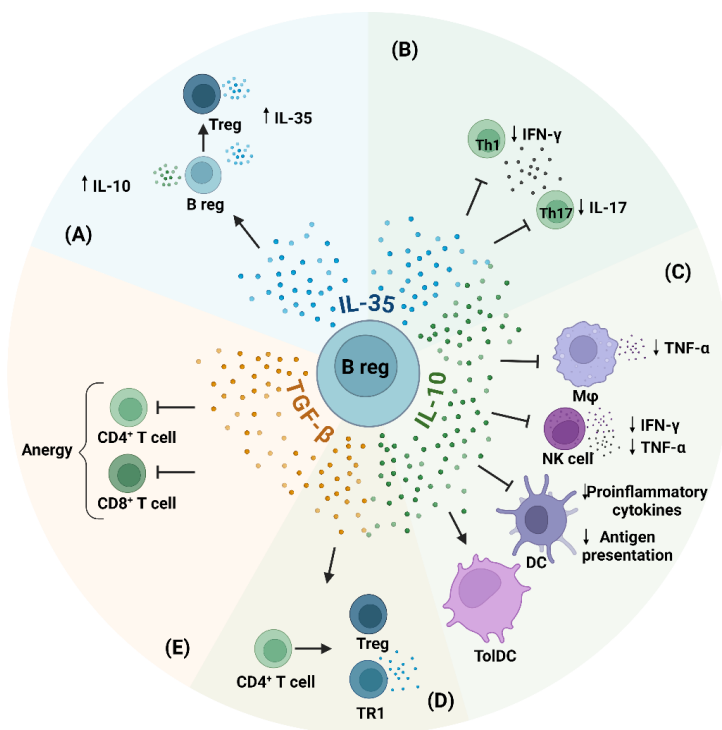


Figure 1. Cytokine-mediated regulatory effects of Bregs. (A) IL-35-producing Bregs promote tolerance by supporting the generation of additional Bregs and inducing IL-35-secreting Tregs. (B) Together with IL-10, Bregs suppress Th1 and Th17 responses leading to reduced secretion of IFN- γ and IL-17, respectively. (C) IL-10 $^{+}$ Bregs modulate M ϕ , Natural Killer cells and DCs by downregulating their proinflammatory cytokine production and the antigen presentation. They also promote TolDCs generation. (D) In conjunction with TGF- β $^{+}$ Bregs, they direct the differentiation of naïve CD4 $^{+}$ T cells into FoxP3 $^{+}$ Tregs or IL-10-producing Tr1 cells. (E) TGF- β release by B regs induces anergy in both CD4 $^{+}$ and CD8 $^{+}$ T cells. Adapted from [380]. Created in BioRender.com.

1.2. Failure of immune tolerance and the development of autoimmune diseases

The dysregulation of the immune tolerance leads to aberrant T cell and/or B cell activity, resulting in inflammation and immune-mediated damage to host cells, tissues or organs, ultimately driving the development of autoimmunity and autoimmune diseases. While the precise mechanisms underlying the breakdown of the immune tolerance remain unclear, it is postulated that immune dysregulation may occur through various mechanisms, including neoepitope formation, chronic inflammatory conditions, molecular mimicry, dysregulated co-stimulatory signalling or dysfunctional regulatory cells [46]. However, autoimmune diseases are not only

the result of this immune imbalance; genetic predisposition and environmental influences also play a role in promoting the loss of tolerance to self. Notably, the incidence of autoimmune diseases has been steadily increasing in recent decades, especially in developed countries, and they have gone from being considered rare diseases to now affecting 5-10% of the population and placing a considerable burden on healthcare systems [47–49]. Although these multifactorial diseases share a common underlying cause— the loss of immune tolerance— they present a diverse array of clinical manifestations depending on the antigen specificity and the nature of the autoreactive cells that initiate the pathogenic immune response. The location of the targeted autoantigen dictates whether the immune response is restricted to a single tissue, as seen in conditions like multiple sclerosis (MS) and immune thrombocytopenia purpura (ITTP), or systemic by affecting multiple tissues, as in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Autoimmune diseases can also be categorized based on the type of autoreactive cells driving the immune attack. For example, myasthenia gravis (MG) is primarily mediated by pathogenic autoantibodies, whereas type 1 diabetes (T1D) is mainly driven by T cell-mediated responses.

1.2.1. Type 1 diabetes

T1D is a chronic metabolic autoimmune disease characterised by hyperglycaemia resulting from the destruction of insulin-producing pancreatic islet β -cells leading to insulin deficiency. Currently affects approximately 8.8 million people globally, with around 510.000 new cases detected per year. Projections suggest a significant rise in T1D prevalence in the coming decades [50]. Interestingly, incidence rates vary substantially across countries partly attributed to improved recognition of the disease due to better access to healthcare services, with the highest rates observed in high-income countries, representing the 49% of the cases. T1D is most commonly diagnosed during childhood and adolescence, but approximately the 30% of the cases are detected in adulthood [51]. The disease develops over a clinically silent period, which can last for months or even years. During this first stage, leukocytic infiltration (insulitis) progressively infiltrates the pancreatic islets of Langerhans, starting the autoimmune attack on β -cells. Concurrently, autoantibodies against different β -cell autoantigens appear in the sera, considered as an early biomarker of β -cell autoimmunity. Stage 2 starts when two or more antibodies are detected, and β -cell mass shows a dysfunction leading to a modest glycaemia associated to impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) [52]. Stage 3 marks the progression to clinical signs, where the decline of β -cell mass is such that endogenous secretion of insulin can no longer meet the metabolic requirements.

This results in overt hyperglycaemia, and patients typically start showing clinical signs of polyuria, polydipsia, polyphagia and substantial weight loss. At this point, T1D can be diagnosed based on a fasting plasma glucose >126 mg/dL or a random plasma glucose >200 mg/dL [53].

Although the exact pathogenesis is still unclear, it is widely believed to result from the interplay of multiple factors, including genetic predisposition, environmental triggers, and immune dysregulation. One of the main components of **genetic risk** is the human leukocyte antigen (HLA) complex, with the DR3-DQ2 and DR4-DQ8 haplotypes being the most strongly associated with T1D and present in 90% of the patients [54]. This association is believed to stem from genetic polymorphisms encoding for different amino acid residues within the peptide-binding pockets of HLA molecules, which in turn affect the binding affinity and diversity of peptides presented to T cells. These variations can lead to the activation of autoreactive T cells, contributing to the T1D development [55]. In addition to the HLA region, genetic studies have expanded the understanding of the genetic basis of T1D, identifying over 60 independent non-HLA candidate genes associated with disease susceptibility [56]. Many of these genes are involved in immune regulation, such as *CTLA4*, *IL2RA* and *protein tyrosine phosphatase nonreceptor type 22 (PTPN22)*, which play roles in modulating the immune response [57–59]. Notably, non-immune-related loci, as the insulin gene (*INS*), are also linked to T1D. A high number of variable numbers of tandem repeats (VNTRs) upstream of the proinsulin gene has been associated with high proinsulin expression in the thymus, potentially enhancing T cell tolerance [60,61]. However, genetic factors alone do not equate to causality, and an array of **environmental factors** are also implicated in triggering seroconversion and disease progression. Viral exposure has been suggested to contribute to β -cell destruction through direct cytotoxic damage and immune tolerance loss [62,63]. Additionally, infant growth and childhood obesity are considered T1D accelerators by promoting systemic chronic inflammation which exacerbates β -cell damage [64,65]. Disruptions in the gut microbiota can lead to enhanced intestinal inflammation and increased gut permeability, potentially promoting β -cell autoimmunity [66].

These factors collectively contribute to the **breakdown of immune tolerance** towards insulin-producing β -cells, where numerous cells of the immune system are involved in both the initiation of pancreatic islet injury and the ongoing immune attack leading to β -cell loss. Initial events comprehend the generation of autoantigen-specific CD4⁺ and CD8⁺ T cells that escape central tolerance mechanisms and stay in the periphery and start surrounding and invading the Langerhans islets [67]. **Innate immune cells** are thought to play an important role in the earliest stages of the T1D. Stressed β -

cells produce chemokines, such as CXC motif chemokine ligand (CXCL)10, chemokine ligand (CCL)2, and CCL20, capable of attracting innate immune cells [68]. Conventional DCs in the pancreatic lymph nodes (PLN) act as professional APCs and, together with inflammatory mediators such as IL-12 and IL-15, may prime the activation of those autoreactive T lymphocytes [69]. M ϕ , one of the most frequent immune cell subsets found in inflamed islets, are thought to contribute to β -cell apoptosis and destruction through the secretion of proinflammatory cytokines, including IL-1 β and IL-6 [70–72]. In addition, defects in phagocytosis processes have been described in patients with T1D, meaning that apoptotic β -cells could not be properly cleared and transformed into secondary necrotic cells, thus further contributing to inflammation [73]. Neutrophils can be found in the exocrine pancreas and may play a pivotal role in the development of T1D by being activated by apoptotic β -cells and sustaining the inflammatory response through proinflammatory cytokines and chemotactic factors release [74,75]. Regarding the natural killer cells (NKs), although present in low numbers in inflamed islets, play a fundamental role in T1D onset [76], with the timing of their presence being a potential indicator of disease progression [77]. However, the precise mechanisms by which NKs contribute to T1D remain unclear.

During the immunological synapse, APCs play a role in modulating the differentiation of **autoreactive T lymphocytes** by secreting specific cytokines. DCs, for instance, drive the activation and differentiation of naïve CD4⁺ T cells into effector subsets such as Th1 or Th17 cells via the production of IL-12 or a combination of IL-6 and IL-23, respectively. Once differentiated, diabetogenic CD4⁺ T cells release cytokines that influence β -cells survival and contribute to their destruction. Th1 lymphocytes, for example, produce interferon-gamma (IFN- γ), which activates M ϕ and stimulates the release of additional proinflammatory cytokines, thereby perpetuating the immunogenic loop [78]. The role of Th17 cells in T1D remains less well-defined; however, some evidence suggests a pathogenic function where IL-17 promotes β -cell apoptosis [79]. Additionally, T helper cells, through cytokines such as IL-2 or IL-21, provide essential signals for the activation of CD8⁺ T cells, which are the predominant immune cell population infiltrating the islets of Langerhans [80,81]. Activated cytotoxic CD8⁺ T cells mediate β -cell destruction through HLA class I-restricted pathways, using mechanisms such as granzyme B secretion and Fas signalling [82]. Furthermore, follicular T helper (Tfh) cells serve as a critical link between autoreactive T and B cells. Autoreactive B cells can directly bind self-antigens through their BCR in secondary lymph organs, process the antigens, and present them via HLA class II molecules to interact with the TCR of Tfh lymphocytes. This interaction results

in reciprocal co-stimulation, leading to the activation, proliferation, and differentiation of autoreactive B cells into memory B cells or autoantibody-secreting plasma cells [83].

Following T1D diagnosis, the unique effective **therapy** for patients is the lifelong exogenous insulin administration, along with strict glucose monitoring via continuous or episodic blood glucose controls. While subcutaneous insulin therapy mitigates complications associated with T1D, patients remain at significant risk for both hyperglycaemic and hypoglycaemic episodes. Advances in technology, such as continuous glucose sensing and smart infusion pumps, have improved glycaemic control and quality of life for many patients [84]. New immunomodulatory strategies aim to go beyond traditional replacement therapy. Monoclonal antibodies (mABs) targeting immune cells or cytokines involved in the autoimmune destruction of β -cells are currently being evaluated in clinical trials. In 2022, teplizumab, an anti-CD3 monoclonal antibody, was approved in the USA as a preventive therapy aimed at delaying the onset of T1D by slowing autoimmunity by temporarily sparing remaining β -cells [85]. These strategies seek to preserve β -cell function and achieve long-term glycaemic control without the exogenous insulin [86–91]. However, their clinical success has been limited, underscoring the need for a deeper understanding of the mechanisms required to effectively translate immunotherapies into meaningful benefits for T1D patients.

1.2.2. Myasthenia Gravis

MG is a rare autoimmune neuromuscular disorder characterized by pathogenic autoantibody-producing B cell-mediated destruction of the components of the postsynaptic membranes in the neuromuscular junctions (NMJ), impairing the communication between motor neurons and muscles [92]. This impairment results in a fluctuating weakness in voluntary muscles, ranging in severity from mild ocular symptoms to pronounced muscle weakness affecting the limbs, bulbar region, and respiratory system. Symptoms can differ widely among individuals, making MG a highly polymorphic condition that can impact physical function significantly and, in severe cases, even become life-threatening. The incidence of MG has risen considerably over the last years, and the number of diagnosed patients has doubled in the last two decades. Prevalence rates have also increased accordingly and now range between 150 and 200 cases per million. This is largely attributed to the increased incidence of MG among elderly, likely due to advancements in diagnostic techniques, improved treatment options and greater population longevity [93]. Interestingly, MG incidence displays a bimodal distribution in women, with peaks

around the ages of 30 and 50. In contrast, incidence rates in men rise steadily with age, reaching their highest levels between 60 and 89 years [92].

MG is rarely inherited, with only about 5% of cases reporting a family history, typically following an autosomal dominant inheritance pattern [94,95]. However, many patients have a personal or family history of other autoimmune disorders— most notably thyroid disease, RA, and T1D— suggesting a potential **genetic predisposition** to autoimmune conditions [96–98]. Recent studies have identified specific HLA alleles implicated in MG susceptibility in different ethnic groups. In Caucasian populations, the HLA A1-B8-DR3-DQ2 haplotype, also known as AH8.1, is significantly associated with the early onset of MG [99,100]. Distinct associations are observed in Asian populations; for example, the DQ9 haplotype is associated with childhood-onset MG in Southern Han Chinese individuals, while the HLA-DRB1(*)09 haplotype is significantly more prevalent in a northern Han Chinese population [101,102]. Additionally, specific alleles, such as DRB1*15:01, DQB1*05:02 and DRB1*16 are linked to an increase of risk for MG late onset in Norwegian and Italian cohorts [103,104]. In addition to HLA-related genes, genetic variants in other genes have also been implicated in MG susceptibility. Variants in the *PTPN22* or TNFAIP3 interacting protein 1 (*TNIP1*) are remarkable examples [105]. Also, single nucleotide polymorphisms (SNPs) within the promoter region of *CTLA4* have been associated with not only MG but also with a broader spectrum of autoimmune diseases, postulating that aberrant splicing or cellular abnormalities of T cells may contribute to autoimmune diseases pathogenesis [57,106,107]. Furthermore, polymorphisms in the alpha (*CHNRA1*) and delta (*CHRNA1*) subunits of the neuromuscular acetylcholine receptor (AChR) complex have shown to confer increased risk of MG [108,109]. While these genetic predispositions increase an individual's susceptibility to MG, **environmental triggers** are often necessary to initiate the autoimmune process. Factors such as emotional stress, physical trauma, exposure to certain drugs or toxins, and viral infections, including hepatitis C, herpes simplex virus, and Epstein-Barr virus, have all been suggested as potential triggering factors in MG development [110,111]

MG patients are classified into subtypes based on serum antibodies and clinical presentation, aiding in tailored therapy and prognostic assessment. Current guidelines and consensus reports advocate for subgrouping, though definitions vary and continue to evolve as new subtypes emerge with advancing research (**Table 1**). Antibodies against AChR, muscle-specific kinase (MuSK), and low-density lipoprotein receptor-related protein 4 (LRP4) are recognized as highly sensitive and specific diagnostic markers and play a key role in defining subgroups. The **AChR antibody-**

associated subtype is the most common, representing the 85% of cases [112]. Nicotinic AChRs are located on the surface of muscle cells, specifically concentrated at the synapse between nerve terminals and muscle fibres, where the impulse transmission happens. Structurally, AChR is a heteropentamer, composed of two α -subunits and one each of β -, δ -subunit, and ϵ -subunit in adult form, or γ -subunit in embryonic form, arranged into a central pore that forms a membrane-crossing channel [113]. Upon acetylcholine release and binding to the α -subunits, the receptor undergoes a conformational change, opening the channel to allow positively charged ions to cross the membrane. This ion flow generates endplate potentials that ultimately lead to muscle contraction. At least half of the AChR autoantibodies are directed against the α -subunits, and they are believed to be the most pathogenic [114,115]. This is likely due to α -subunit's prominent location within the receptor, which leaves it more accessible to the antibodies, as well as its role in modulating the receptor sensitivity to AChR binding [116,117]. This group of patients present reduced endplate potential due to a pathologically low density of AChR, which is translated into the clinical manifestation of muscle weakness. The pathogenic mechanisms and functional spectrum of the autoantibodies are diverse; they may damage the post-synaptic membrane through complement cascade activation or disrupt AChR functionality by binding, blocking or modulating its activity [118–120]. The extraocular muscles are predominantly impacted in this disease, as they require sustained tonic contractions to maintain the eye positioning, with muscle fibers exhibiting a high frequency of synaptic firing, and quicker tension development. Additionally, these muscles have lower AChR density, making them more susceptible to the effects of receptor depletion and thus contributing to the high prevalence of ocular symptoms at the onset [121]. Notably, about 15% of AChR-MG patients, two years post-diagnosis, present only ocular symptoms without further systemic manifestations and are thus classified under the ocular AChR-MG subtype. The rest of the patients progress to systemic manifestations and are classified as generalised AChR-MG and being subdivided based on the age of the onset. Early-onset AChR-MG typically occurs in patients under 50 years old, with a female-to-male ratio of 3:1. This subgroup shows a higher incidence of thymic follicular hyperplasia and an association with other autoimmune diseases [98,122]. In contrast, late-onset AChR-MG, defined by onset after the age of 50, shows no female predominance; instead, a slightly higher prevalence is observed among men over 60, and thymic hyperplasia is rare in this group [123]. Another subtype of AChR-MG is associated with thymoma, a condition presenting an abnormal development of critical epithelial cells within the thymus. This leads to a breakdown in intrathymic tolerance that results in the formation of autoreactive T cells that cannot be

effectively deleted [124]. This increased self-reactivity may also explain the frequent presence of antibodies against other NMJ components, such as titin or ryanodine.

Among the **non-AChR-MG subtypes**, patients with MuSK autoantibodies represent approximately 4% of all MG cases: notably, cases with concurrent seropositivity for both AChR and MuSK antibodies are rare [125]. MuSK itself is an essential membrane protein that promotes the clustering of AChRs at the NMJ. Upon release from the presynaptic terminal, agrin protein binds to Lrp4 protein, triggering the reorientation of the Lrp4/MuSK complex and activating MuSK through phosphorylation. This activates a downstream signalling cascade that clusters AChRs, stabilizing the NMJ [126]. The pathogenic action of MuSK antibodies is mediated through binding to the Ig-like domain, which prevents MuSK phosphorylation and disrupts the agrin-Lrp4-MuSK signalling pathway, which is critical for NMJ stability [127]. This subtype shows a pronounced female predominance, with up to 85% of MuSK-positive patients being female [128]. Clinically, these patients usually present severe clinical symptoms, particularly affecting the facial, bulbar, and respiratory muscles, although ocular symptoms are uncommon. In contrast, patients with LRP4 antibodies represent around the 2% of the MG cases, also showing a female predominance but generally with milder clinical signs [129]. LRP4 is a proteoglycan molecule part of a recently identified family of proteins that serves as the receptor for neural agrin, thereby playing a crucial role in the agrin-dependent activation of MuSK at the NMJ [126]. The pathogenicity of LRP4 antibodies lies in their interference with MuSK activation, disruption the signalling pathway necessary for AChR clustering [130]. Finally, a minor group of patients which are classified as triple seronegative, as they lack detectable AChR, MuSK, or LRP4 autoantibodies. However, these patients may harbour antibodies against other complexes, such as titin or ryanodine receptors, or potentially against yet-unidentified antigens, including those targeting clustered proteins at the NMJ [131]. Clinically, their presentation closely resembles that of AChR-positive MG patients, particularly in terms of treatment response and thymic abnormalities, suggesting similar underlying mechanisms despite the absence of conventional autoantibodies [132].

Table 1: Subtype classification of MG patients based on autoantibodies specificity

Autoantibodies	Clinical manifestation	Age of onset	Relative prevalence	Targeted population	Thymic pathology
AChR	Ocular	>50	80-85%	Adult in USA and Europe; childhood in Asia	Unknown
	Generalised early onset	<50		Females	Follicular hyperplasia
	Generalised late onset	>50		Males	Normal
	Thymoma-associated	>50		40-60	Neoplasia
MuSK	Cranial and bulbar muscles	<40	~ 4%	Young females	No
LRP4	Facial, bulbar, and respiratory muscles but not ocular	<50	~ 2%	Young females	No
Seronegative	Generalised	<40	~ 5%	Young individuals	Mild follicular hyperplasia

AChR, acetylcholine receptor; LRP4, low-density lipoprotein receptor-related protein 4; Musk, muscle-specific kinase.

The clinical signs of MG are mediated by autoantibodies, but their production is largely dependent on AChR-reactive T cells, thus implicating defects in B- and T-lymphocyte tolerance in the onset of the disease. Notably, both new emigrant and mature B cells expressing polyreactive or autoreactive BCRs are increased in those patients with both AChR-MG and MuSK-MG subtypes [133]. Although AChR-reactive T cells can be detected in the bloodstream of healthy individuals, they normally remain inactive by peripheral tolerance mechanisms. In early onset MG, however, this population of potentially autoreactive T cells is likely involved in disease development. In addition, thymic hyperplasia or thymomas contribute to generate and release autoreactive CD4⁺ and CD8⁺ T cells which are typically excluded from the peripheral T cell repertoire. This is due to the aberrant inflammatory conditions and intratumoral microenvironment where thymocytes are selected and matured under abnormal conditions [134]. Moreover, other molecular components are deficient in thymomas, including AIRE or FoxP3 [124]. Furthermore, the resulting T cell subset imbalance and cytokine dysregulation promotes B cell activation within germinal centers and differentiation into autoreactive B cells and plasma cells [135]. Cao and colleagues detected that autoreactive T cells in MG patients presented elevated

production of IL-17, IFN- γ , and granulocyte-macrophage colony-stimulating factor and sargramostim (GM-CSF), alongside a reduced IL-10 production, reflecting the key imbalance between autoreactive cells and regulatory cell types, a hallmark commonly associated with autoimmune diseases [136]. In this sense, studies have shown that CD4⁺ Tregs from thymus and peripheral blood cells of MG patients had an impaired suppressor function despite the number of CD4⁺ Tregs was constant [137–139]. Additionally, MG patients show a reduced frequency of regulatory B cell subsets, such as CD19⁺CD1d^{hi}CD5⁺ and CD19⁺CD24^{hi}CD38^{hi} cells, including IL-10-producing B cells within these groups [130–133]. Overall, the expansion of Tregs and Bregs or the restoration of its suppressive function represents a potential therapeutic strategy in MG.

First-line **treatment** for MG primarily involves symptomatic drug therapies, including acetylcholinesterase inhibitors, which effectively increase acetylcholine levels at NMJ and have demonstrated to be the most effective symptomatic treatments in MG at initial stages [140]. For patients in all subgroups who do not have a fully satisfactory functional outcome with symptomatic treatment alone, immunosuppressive drugs are used. For short-term immunosuppression, corticosteroids are commonly used to inhibit T cell activation, reduce inflammatory effects of monocytes and M ϕ , and to reduce the size and numbers of germinal centers in the thymus [141]. However, their long-term use is associated with a wide range of adverse effects. Long-term immunosuppression is used when corticosteroids do not present good clinical effects, in this group are included DNA synthesis and T cell proliferation inhibitors such as cyclosporine, cyclophosphamide and methotrexate. Rituximab, chimeric monoclonal antibody against the B cell surface marker CD20, has demonstrated efficacy in some clinical cases with a positive outcome [142]. Recently approved CD5 inhibitors, such as Eculizumab or Ravulizumab, a recombinant humanised monoclonal anti-CD5 antibodies, have shown efficacy in preventing complement activation [143,144]. The neonatal Fc receptor (FcRn) antagonist strategy has shown efficacy in reducing IgG levels, including pathogenic types, by accelerating their catabolism, thus achieving a therapeutic impact [145,146]. On the other hand, intravenous (IV) administration of immunoglobulins purified from human blood or plasma exchange can be used as a rapid short-term immunomodulation in severe crisis cases when patients need for intubation for respiratory. Although the mechanism of action is not completely understood, it is postulated that key mechanisms include interference of signalling via Fc receptors, neutralization of activated complement, suppression of idiotypic antibodies, and modulation of proinflammatory cytokines [147–149].

Finally, thymectomy, for patients with thymoma or early-onset AChR-MG patients, is highly recommended after the first clinical signs detected. The mechanism by which thymectomy potentially benefits non-thymomatous patients with generalized MG is incompletely understood but may involve disrupting B cells that produce the autoantibodies. The introduction of these treatments has significantly lowered MG-related mortality rates from the early 20th century, although mortality remains at 5-9% with slightly higher rates in males than females [110].

1.2.3. Rheumatoid Arthritis

RA is a chronic systemic autoimmune disorder that predominantly affects the joints. However, it also has extra-articular manifestations such as rheumatoid nodules, pulmonary involvement or vasculitis, and systemic comorbidities such as cardiovascular disease, being the most common cause of premature death in people with RA [150]. The disease is hallmarked by the production of pathogenic antibodies directed against IgG, described as rheumatoid factor (RF), and citrullinated proteins, anti-citrullinated protein antibodies (ACPAs). RA is associated with progressive disability by accumulating joint damage, premature death and high socioeconomic burdens [151,152]. Its prevalence differs across ethnic groups, ranging from 2.500 to 10.000 cases per million individuals [153]. Although RA can develop at any age, its incidence increases after the age of 40, with women being affected two to three times more often than men [154].

The RA pathogenesis is complex and is not fully understood, however, it has a strong **genetic component**, since there is a two to five times higher risk of developing RA compared to the general population when a first-degree relative is affected [155]. The HLA system shows a strong influence in the pathogenesis, especially the HLA-DRB1*01 and HLA-DRB1*04 alleles, which have been described as the most associated with the risk of developing RA [156,157]. Over 100 loci across the genome have been identified as contributing to RA susceptibility, with many of these genes involved in the immune system regulation. Notable examples include *PTPN22*, *CTLA4*, *PADI4*, *TNIP2*, *WISP1* and *TNFRSF11A*, although each individual locus provides only a modest contribution to disease risk [158–160]. In addition, **epigenetic mechanisms** are thought to have a role in the pathogenesis. Studies indicate that some genetic variants associated with RA are enriched in epigenetic marks of active chromatin in CD4⁺ T helper cells [161]. Furthermore, altered histone acetylation and DNA methylation demonstrated to regulate the function of synovial fibroblasts and leucocytes [162]. These epigenetic modifications offer a mechanism through which

environmental factors can induce cellular activity, such as smoking, dust inhalation, low socioeconomic status or the microbiota [163–166].

The **pathogenesis course** of RA begins with a high-risk or susceptibility phase driven predominantly by genetic predisposition and perpetuated through environmental factors (**Figure 2**). Next stage is termed preclinical RA and encompasses several years before clinical joint inflammation develops. The precise location of immune system activation is uncertain, but evidence suggest that it is distant from the joint. The lungs are a strong candidate, given the well-established negative impact of cigarette smoke on RA risk and the identification of shared citrullinated peptides in both lung and synovial tissue biopsies [167]. Other mucosal sites, such as the periodontium and the gut also have been proposed to be involved where microbiota may participate in the early initiation of the RA [168]. At these mucosal sites, cellular stress triggers citrullination, a post-translational modification of a positively charged arginine into a polar uncharged citrulline, of a wide range of proteins, including vimentin, α -enolase, fibronectin, fibrinogen, histones, and type II collagen. This process can influence hydrogen bonding and ionic interplay, and therefore interferes with organized protein structure, possibly leading to destabilization or even suppression of inter- and intramolecular interactions [169]. These altered peptides bind to MHC heterodimers, presenting citrullinated antigens to T cells, which in turn stimulate B cells to produce autoantibodies including RF and ACPAs, being detectable a decade before clinical diagnosis [170]. However, the presence of ACPAs alone is not sufficient to induce synovitis, which is characterized by increased vascular permeability and inflammatory cell influx into the synovium. An additional trigger is likely required to initiate the clinical synovitis such as the formation of circulating immune complexes containing citrullinated peptides. These complexes can enter to the joint, depositing on cartilage and synovial tissue, where they activate M ϕ via the synergistic engagement of TLR4 and Fc receptors or through complement activation. This cascade triggers a secondary inflammation, initiating the transition into the early RA stage [171]. Cytokine and chemokine networks play a critical role in the pathogenesis, aggravating inflammation and sustaining the immunogenic loop [172]. The cellular composition of the synovitis is dominated by CD4⁺ T cells and M ϕ , together with early stromal cell activation. CD4⁺ memory T cells can form ectopic germinal centres providing a microenvironment where B cells proliferate, differentiate and produce antibodies. Within these centres, B cells undergo affinity maturation, further expanding the ACPAs repertoire [173]. M ϕ , neutrophils and mast cells contribute to joint damage through the release of proinflammatory cytokines, chemokines and growth factors. These mediators can activate local fibroblast-like-

synoviocytes (FLS), driving the production of proteolytic enzymes such as matrix metalloproteinases (MMPs), collagenases and stromelysins [174]. This aggressive subset presents increased invasiveness and may migrate between joints perpetuating disease progression. Activated FLSs and M ϕ also produce TNF, IL-1 and IL-6, which, in conjunction with activated T cells, B cells and autoantibodies, promote osteoclast maturation and activation, a process mediated by receptor activator of nuclear factor κ B ligand (RANKL), macrophage colony stimulating factor (M-CSF), TNF and sialic acid-binding immunoglobulin-like lectin (Siglec)-9 [175–177]. Osteoclasts degrade the mineralized bone matrix by producing proteases, including cathepsin K, within a specialized acidified local tissue microenvironment. Meanwhile, cartilage damage is driven by catabolic activity in chondrocytes, which are stimulated by proinflammatory cytokines, further contributing to joint destruction, a hallmark of established RA disease [178].

In routine practice, the diagnose of RA often relies on the classification criteria established by the American College of Rheumatology (ACR) and European Alliance of Associations for Rheumatology (EULAR) [179]. Early intervention, particularly within the first three months following the diagnosis, is crucial for long-term disease management. Initiating **disease-modifying antirheumatic drugs (DMARDs)** early has been shown to be key for improving prognosis and outcomes over the course of the disease [180,181]. DMARDs are categorised into three main groups. The first group, conventional synthetic DMARDs, comprises small-molecular-weight synthetic drugs with unclear anti-inflammatory mechanisms. Methotrexate is the most commonly used, particularly for patients with moderate-to-high disease activity. However, despite its efficacy, it is associated with low tolerability, low compliance and safety issues [182]. For patients with low disease activity, hydroxychloroquine or sulfasalazine are preferred due to their tolerability and safety profiles. The second group encompasses biological DMARDs, including mostly mABs or, less often, soluble receptor constructs, with diverse mechanisms of action. These range from the inhibition of TNF- α and IL-6 receptor antagonism to the blockade of T cell co-stimulation and B cell depletion. While these biological agents are effective, they are associated with a higher risk of adverse events, including an increased susceptibility to infections. Lastly, the third group, targeted synthetic DMARDs, focuses on inhibiting specific intracellular enzymes, such as Janus kinase (JAK) inhibitors, thereby blocking the production of multiple cytokines implicated in the pathogenesis of the disease. These agents have proven higher efficacy but are also linked to side-effects, including an increased risk of herpes virus reactivation and venous thromboembolism [183].

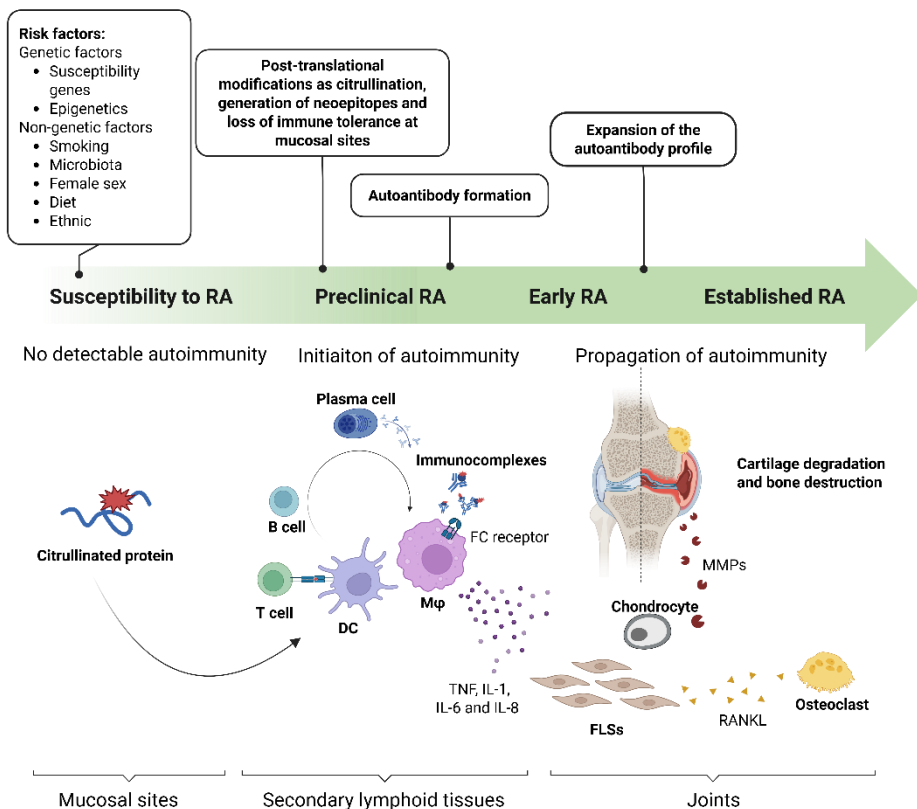


Figure 2. Mechanisms involved in development and progression of RA. This autoimmune condition arises from a combination of genetic and non-genetic risk factors, with disease development likely requiring the accumulation of multiple triggers before reaching a critical threshold. The progression of RA begins with the initiation and propagation of autoimmunity against citrullinated self-antigens, a process that may happen years before clinical signs emerge. These altered peptides are presented by APCs, activating adaptive immune response and driving the production of autoantibodies. Locally, stromal cells such as fibroblast-like synoviocytes (FLS), APCs and Mφ become activated, contributing to the production of inflammatory mediators. This autoimmune response results in persistent synovial inflammation, maintained by the continuous formation of immune complexes. Over time, this chronic inflammatory environment leads to the progressive destruction of cartilage and bone, hallmarks of established RA pathology. Adapted from [142]. Created in Biorender.com.

1.2.4. Immune Thrombotic Thrombocytopenic Purpura

Immune thrombotic thrombocytopenic purpura (iTTP) is a rare, but potentially life-threatening autoimmune blood disorder. It is caused by the production of autoantibodies against a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13 (ADAMTS13), resulting in a severe deficiency of more than 90% of its usual activity [184]. ADAMTS13 is mainly synthesized in the liver by hepatic stellate

cells, and it is released as an active enzyme in the bloodstream [185]. Its main function is the rapid cleavage of ultra-large newly secreted von Willebrand factor (VWF) polymers by the endothelial cells. In the bloodstream, the low activity of ADAMTS13 results in an enhanced platelet aggregation and spontaneous thrombi formation in small arterioles and capillaries. Hence, iTTP patients suffer from systemic clumping of platelets leading to systemic organ damage, marked thrombocytopenia (low blood platelet count) and red blood cell disruption [186]. iTTP is mostly presented in adult patients with peak incidences in the third or fifth decades, and female to male ratios ranging from 2.5:1 to 3.5:1 [187]. The prevalence rates varied depending on the ethnicity but ranged between 13-18 cases per million [188].

Evidence suggests a **genetic predisposition** in the development of autoantibodies against ADAMTS13 in iTTP. The involvement of the HLA system is well-documented, with the HLA-DRB1*11 allele being overrepresented in iTTP patients, while HLA-DRB1*04 appears to have a protective role, as it is notably absent in affected individuals [189,190]. Additionally, other genetic factors, such as SNPs of genes encoding for (*TLR-9*) and *PTPN22* have been implicated in the development of iTTP and with other autoimmune diseases [59,158,159,191,192]. Despite the presence of genetic risk alleles, not all carriers develop the corresponding autoimmune disease, underscoring the interplay between genetic predisposition and environmental factors. Post-translational modifications of antigens, such as glycosylation changes in ADAMTS13, can alter antigen presentation in HLA risk alleles or modify TCR recognition, contributing to autoimmunity [193,194]. Citrullination may also play a role as ADAMTS13 can be citrullinated under stress conditions, a process previously described as a trigger in RA [170,195]. As with many autoimmune diseases, the aetiology of iTTP reflects a complex interplay between genetic disposition, the immune system, and **environmental factors**. The development of ADAMTS13 autoimmunity may be associated with bacterial or viral infections, which are frequently associated with disease onset. Common triggers include HIV, Hepatitis C and influenza A viruses. The role of microbiota has also been proposed, given its dependence on geography, ethnicity, and lifestyle, with significant variations worldwide [196]. Dysbiosis, defined as the imbalance in bacterial composition, may lead to the presentation of microbiota-derived antigens, which, through molecular biomimicry, could trigger the autoimmune reaction against self-antigens in those genetically susceptible individuals [197]. Additionally, some reports suggested an association between vaccine administration and iTTP onset, such as following the influenza vaccine [198].

Independently of the initial trigger, the iTTP onset requires the uptake of ADAMTS13 by APCs. These APCs process and present in an immunogenic fashion ADAMTS13 derived peptides through HLA-class II molecules. Notably, proteomic studies examining HLA-DR and HLA-DQ-presented peptides have revealed that most of the eluted peptides are derived from the CUB2 domain of ADAMTS13 [199]. The strong association of HLA class II risk alleles with iTTP, coupled with the presentation of ADAMTS13-derived peptides, underscores the critical **role of CD4⁺ T cells** in the immunopathogenesis of this disease. Subclass and clonal analyses of anti-ADAMTS1 antibodies from patients further support this role, as they demonstrate evidence of isotype switching and affinity maturation [200]. These processes further corroborate the role of CD4⁺ T cells in the autoimmune progression against ADAMTS13. These immunological processes suggest that autoreactive CD4⁺ T cells differentiate into effector phenotypes, producing proinflammatory cytokines and subsequently stimulating antigen-specific B cells to differentiate into plasma cells, which secrete pathogenic anti-ADAMTS13 autoantibodies. Some produced antibodies have demonstrated the ability to target SPACER domain of ADAMTS13 protein and modify it to an open conformation, also related with its activity. Moreover, this open conformation is correlated with iTTP disease severity and relapse, serving as a biomarker of disease progression [201].

A rapid diagnosis and early **treatment** in patients with suspected iTTP are critical to prevent significant morbidity and mortality. Therapeutic plasma exchange (TPE) forms the cornerstone of acute-phase treatment, as it replenishes circulating ADAMTS13 levels while removing anti-ADAMTS13 autoantibodies [202]. Immunotherapy complements TPE to target autoantibody production are combined, enabling the recovery of ADAMTS13 activity. Corticosteroids can be used as first-line immunosuppressive agents, and rituximab is frequently administered during the acute phase, either concomitantly or shortly after TPE, to reduce relapse rates [203]. For patients with contraindications to steroids or refractory disease, alternative immunosuppressive therapies such as cyclosporine A, mycophenolate mofetil, or cyclophosphamide have shown efficacy [204]. Emerging therapies targeting VWF have also demonstrated promise. Caplacizumab, a humanized immunoglobulin originally derived from llamas that binds the A1 domain of VWF and prevents its interaction with platelets. This approved therapy, is implemented in the triplet therapeutic strategy for its significant role in reducing the time to platelet count normalization and mitigates end-organ damage [205]. Additional anti-VWF strategies are under development with the goal of blocking platelet binding via the VWF A1 domain. However, these approaches require concomitant immunosuppression to

address the underlying autoimmune deficiency of ADAMTS13. Patients are at an elevated risk of premature mortality, primarily due to cardiovascular disease, emphasizing the need for continued research and the development of novel therapies to improve outcomes [206].

2. New treatments for autoimmune disorders: Induction of immune tolerance

As aforementioned in chapter 1, **conventional treatments** for autoimmune diseases primarily rely on replacement therapies, symptomatic drugs or broad-spectrum immunosuppressive drugs designed to alleviate disease severity. While these agents are often effective and serve as a first-line treatment at the disease onset, they do not target the underlying mechanisms driving autoimmunity. Moreover, as a long-term treatment, they may expose the patient to side effects, cumulative toxicity, potential life-threatening opportunistic infections and higher risk of appearance of malignancies.

The introduction of immunomodulatory strategies, commonly referred as **biologics**, achieved to minimize the side effects associated with traditional broad-spectrum immunosuppression. These therapies primarily use mAbs that selectively target proinflammatory cytokines, key inflammatory mediators, or specific leukocyte populations involved in autoimmune responses [207]. Among cytokine-targeting mAbs, those directed against TNF- α , IL-1, IL-6R and against the IL-12, IL-23, IL-17 axis have shown efficacy in a limited number of autoimmune diseases [208–211]. However, the complex hierarchy of cytokines and the diversity of patient endotypes make their broad application challenging. Following the initial success of targeting specific leukocyte subsets in lymphoproliferative disorders, mAbs such as those targeting CD20, CD3 or CD52 have demonstrated efficacy by depleting autoantigen-specific secreting B cells, mature T cells, or both [212–214]. Despite their effectiveness in certain patient populations, these therapies often cause severe side effects proportional to the degree of immunosuppression. Another approach involves inhibiting leukocyte migration by targeting adhesion molecules such as CD11a and CD49b or chemokine receptors like CXC chemokine receptor (CXCR)-3 and CCR6 [215–218]. By preventing the recruitment and activation of effector T cells at inflammatory sites, these strategies reduce localized inflammation but can lead to severe adverse effects, such as the reactivation of opportunistic viral infections [219,220]. Additionally, mAbs targeting co-stimulatory or coinhibitory immune checkpoints have gained attention. These agents either block pathways that promote

T cell activation, such as CD2, CD28, CD40L, and inducible T cell co-stimulator (ICOS), or enhance inhibitory signals through molecules like BTLA, LAG-3, T cell immunoglobulin and mucin-domain containing-3 (TIM-3), TIGIT, PD-1/PD-L1, and V-domain Ig suppressor of T cell activation (VISTA) [221–227]. Although biologic strategies demonstrate good tolerability and better safety profiles, they do not provide a robust and long-lasting immune regulation and not direct toward the root cause of these diseases necessary for curative interventions. Moreover, mAb therapies can induce the formation of anti-drug antibodies (ADAs), which may impact treatment efficacy and safety [228].

Self-tolerance is thought to be maintained not only by promoting the deletion of autoreactive lymphocytes but also by enhancing the number or function of regulatory lymphocyte subsets. Recently, therapies aiming to expand the patient's pools of regulatory T cells to counteract antigen-specific immune activation, have also made their way into the clinical field. Low-dose IL-2 therapy has demonstrated a safety profile and the ability to selectively expand CD25 expressing Treg cells *in vivo* without significantly affecting other immune cell subsets [88,229]. Another promising approach involves **cell-based therapies**, as the *in vitro* expansion of polyclonal FoxP3⁺ Tregs or Tr1 cells, followed by their reinfusion into patients [230,231]. The safety and tolerability of these broadly acting strategies are acceptable; however, their effectiveness remains limited due to the transient effects and the reduced survival capacity of the transferred cells [232]. The potential use of **chimeric antigen receptor (CAR) T cells** for the treatment of autoimmune diseases is seen as a breakthrough in the field, but their use is associated with high production costs and significant gaps in their safety profile, including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) [233].

2.1. Therapeutic induction of antigen-specific immune tolerance

Due to the lack of an effective treatments achieving a long-term efficacy by targeting the fundamental cause of the autoimmune diseases—the breach of tolerance to self-antigens— it is logical to speculate that the fine-tuning of antigen-specific immune tolerance strategies would represent a better approach to tackle autoimmune disorders. These innovative approaches, called antigen-specific immunotherapies (ASIT), are designed to suppress pathogenic immune cells while preserving protective immunity, providing the potential for durable and disease-specific treatment. Current strategies fall into four main categories (**Figure 3**): DNA-based therapies, which use plasmids or viral vectors encoding self-antigens; peptide-based therapies, involving the administration of disease-specific peptides to tolerize T cells; cell-based

therapies, which expand or modify regulatory immune cells *ex vivo* for reinfusion; and nanoparticle (NP)-based therapies, where antigens are encapsulated in to direct tolerogenic immune responses. Together, these approaches represent a transformative shift toward precision medicine in the treatment of autoimmune diseases, with the potential to achieve long-lasting remission.

2.1.1. DNA-based strategies

Approaches based on DNA are an attractive method for antigen-specific modulation due to their straightforward manufacturing process. Several companies are exploring this strategy by developing **plasmid-based vaccines** encoding disease-associated antigens. A critical feature of these plasmids is the removal of TLR-9 activating CpG motifs to minimize innate immunity activation. Tolerion (<https://tolerion.bio>) used this strategy administering a plasmid encoding for the full proinsulin molecule or the myelin basic protein. While in clinical trials this strategy showed a favourable safety profile, trials targeting T1D and MS failed to achieve their clinical endpoints [234,235]. To enhance efficacy, improved strategies are under development. For example, AditXt (<https://www.aditxt.com>) designed the co-administration of plasmids encoding for GAD65 and the immunomodulator pro-apoptotic BAX protein, which has shown promise in experimental models by inducing tolerogenic DC migration to lymph nodes, suppressing autoreactive T cell proliferation, and increasing anti-inflammatory cytokine production [236]. Another approach developed by Novo Nordisk (<https://www.novonordisk.com>), currently in phase I clinical trials, involves a multicomponent plasmid encoding pre-proinsulin, TGF- β , IL-10, and IL-2. This strategy promotes antigen-specific Treg generation and increased peripheral levels of endogenous IL-10 [237]. Despite the advancements, these strategies face challenges related to the control of half-life, biodistribution, and cellular uptake of the plasmid products. To address these limitations, **viral vectors** such as adenovirus, lentivirus, and plant viruses are being explored as delivery systems [238–240]. However, these approaches introduce potential genotoxicity risks, which must be carefully managed.

2.1.2. Peptide-based strategies

The induction of immune tolerance through the administration of **whole proteins** or autoantigens has emerged as a cornerstone in the development of immunotherapies to tackle autoimmune settings. Although several of these approaches have reached clinical trials and demonstrated favourable safety profiles, their efficacy has often fallen short of the benchmarks required for progression to later clinical stages [241].

Refined therapeutic strategies using **peptides** to modulate the antigen-specific responses have been developed. For instance, in celiac disease, one approach involved administering three immunodominant peptides specific to gluten-reactive CD4⁺ T cells. This strategy aimed to induce unresponsiveness in autoreactive T cells following gluten re-stimulation. While this approach advanced to a Phase I clinical trial, it was ultimately discontinued [242]. Similarly, effort to treat MS have focused on inducing antigen-specific tolerance through intradermal administration of a mixture of three myelin-derived peptides, resulting in the generation of Tr1 regulatory T cells [243]. By contrast, a novel alternative has been developed by Worg Pharmaceuticals (<https://www.worgpharma.com>), using their apitope discovery platform. This approach involves designing short peptides capable of directly binding to MHC molecules on APCs in an antigen-processing-independent manner. This binding occurs in the absence of co-stimulatory signals, promoting the induction of antigen-specific anergic and regulatory T cells [244]. Currently, this strategy is under clinical evaluation for MS and Grave's disease [245,246]. Curavac (<https://www.curavac.com>) uses also algorithmically designed peptides that are mimetics of antigen receptors on autoreactive T and B cells involved in autoimmune pathology. These peptides aim to generate antibodies targeting the autoantigen-receptor complexes, effectively eliminating those autoreactive cells. This approach is currently in phase 2 trials for MG, aiming to prevent disease progression and mitigate symptoms such as muscle fatigue by reducing AChR antibody responses [247,248].

Additionally, a number of strategies have centred on finding the antigen tolerance by the use of **modified peptides**, such as the company Imcyse (<https://www.imcyse.com>), which developed synthetic peptides incorporating a thioreductase motif flanked by class II-restricted T cell epitopes in order to strengthen the T cell receptor-MHC interaction. This approach aimed to stimulate antigen-specific cytolytic effector memory CD4⁺ T cells, which selectively target and lyse APCs presenting the selfantigen:MHC complexes [249–251]. Clinical trials for this strategy, including Phase II trials for T1D and Phase I trials for MS, have been performed despite yielding limited results [252]. As a result of these circumstances, the company ceased its activities in 2024. On the other hand, Toleranzia (<https://www.toleranzia.com>) focuses on the IV administration of a modified extracellular domain of the $\alpha 1$ chain of the AChR. This modified peptide triggers the tolerization of DCs, promoting antigen-specific tolerance [253,254]. The approach is preparing to enter Phase 1 clinical trials for MG. Additionally, Anokion (<https://anokion.com>) has pioneered a liver-targeting mechanism by conjugating autoantigens to synthetic glycopolymers, such as poly(N-acetyl glucosamine)

(p(GluNAc)) or poly(N-acetyl galactosamine) [255]. These conjugates are designed to target hepatic APC subsets which internalize the autoantigens through C-type lectin receptors, facilitating their presentation in a tolerogenic microenvironment [256]. This approach is currently in phase 2 and phase 1 for celiac disease and MS, respectively, showing a good safety and tolerability profile [257].

By contrast, some strategies are focused on the **co-delivery** of peptides together with immunomodulatory agents that help the immune system to interpret the new source of antigen as non-immunogenic. In this category, highlights the company Diamyd (<https://www.diamyd.com>), which started exploring the use of aluminum-formulated glutamic acid decarboxylase 65-kilodalton isoform (GAD65) to suppress type 2 T-helper cells and humoral responses. Although the strategy demonstrated tolerability in early trials, current clinical trials are exploring modifications, such as combining the therapy with immunomodulators like vitamin D, altering the administration route to intranodal injection and modifying the administered dose, to enhance efficacy [258–261].

Despite the promising advancements in peptide-based immunotherapies, challenges remain in achieving the efficacy required for widespread clinical application. Further optimization is necessary to enhance product stability, improve therapeutic efficacy through the use of co-adjuvants, and refine dosing strategies.

2.1.3. Cell-based therapies

Some studies have centred on *ex vivo* cell-based strategies. One commonly used approach involves **deriving monocytes into tolerogenic DCs**, which are then pulsed with the disease-specific autoantigen. Different conditions to tolerise the moDCs have been tested, including the use of anti-inflammatory cytokines such as IL-10 and exogenous mediators like vitamin D3 or dexamethasone. This strategy achieves a tolerogenic phenotype, and the autologous reintroduction of these cells have shown to induce antigen-specific IL-10-producing T cells *in vivo*, Bregs, and the stimulation of autoreactive T cell anergy [262,263]. Clinical trials using these approaches have demonstrated safety in patients with autoimmune disorders such as T1D, RA, MS, NMO and Crohn's disease [264–268]. Similarly, a new strategy uses lentiviral vectors encoding immunodominant antigen-derived peptides and IL-10 to induce antigen-specific IL-10-producing DCs from peripheral monocytes. These *ex vivo* transduced DCs have demonstrated the ability to downregulate autoreactive T cells and induce antigen-specific Tr1 cells in an experimental model of T1D [269]. However, despite their proven efficacy and the well-characterised tolerogenic markers, there is no

standardized method to generate tolerogenic DCs *ex vivo*, which challenges their implementation of these strategies in clinical settings.

Alternatively, strategies using ***ex vivo* lymphocytes and red blood cells** coupled with disease-specific antigens have been employed to achieve tolerance based on the liver's non-immunogenic clearance of dying-cells. For instance, Cellerys (<https://www.cellerys.com>) demonstrated the safety of intravenously reintroducing red blood cells coupled with myelin peptides into MS patients in a Phase I clinical trial [270]. A novel microfluidic loading technique, which temporarily disrupts cell membranes to load antigens, have shown efficacy in generating antigen-loaded red blood cells. These cells can increase FoxP3⁺ T cells in the spleen after reintroduction and induce Treg bystander suppression of polyclonal autoreactive T cells were also described in experimental model of T1D [271,272]. Tolerogenixx (<https://www.tolerogenixx.com>) initially developed its strategy to prevent transplant rejection but has recently extended it to treat autoimmune disorders [273]. This approach involves *ex vivo* treatment of peripheral blood mononuclear cells with mitomycin C, inducing a non-immunogenic apoptotic process, followed by reinfusion on the same day [274–276]. However, the company has not yet disclosed specific details regarding its autoimmune disease approach.

Recently, genetic engineering approaches to produce **chimeric antigen-specific receptor (CAR)-Tregs** have shown potential for enhanced treatment efficacy and reduced systemic side effects in autoimmune conditions. In an experimental autoimmune encephalomyelitis (EAE) model, CD4⁺ CAR/FoxP3⁺ Tregs suppressed disease by reducing symptoms and proinflammatory cytokine secretion in inflamed tissues [277]. Similarly, inducing myelin basic protein-specific T cell clone expression in human Tregs derived from peripheral blood has demonstrated the ability to suppress autoreactive T cells. Importantly, these engineered Tregs also maintained tolerogenic stability under pro-inflammatory conditions [278]. Despite these promising results, challenges such as high production costs and the risk of immunological toxicities remain significant barriers to advancing these therapies into clinical trials.

2.1.4. Nanoparticles strategies

Recent advancements in immunotherapy have introduced NP-based strategies as a promising alternative. NPs acts as a versatile delivery vehicle, capable of presenting autoantigens in a controlled and targeted manner. Unlike *ex vivo* cell-based therapies, NPs offer scalable, cost-effective production while overcoming the

logistical challenges associated with cell manipulation. Furthermore, NPs protect antigens from degradation, ensuring stability and prolonged bioavailability. Among the most widely used are **polymeric NPs**, which are relatively simple to manufacture, fine-tuned to meet certain characteristics, and quickly degraded. However, some polymeric materials can activate immunostimulatory pathways, and usually the carried peptides are exposed on their surface, exposing them to proteolytic cleavage and therefore impacting in their quality [279]. For instance, COUR Pharmaceuticals (<https://courpharma.com>) has developed negatively charged poly(d,l-lactic-co-glycolic acid) (PLGA) NPs encapsulating disease-specific autoantigens [280]. For instance, COUR Pharmaceuticals (<https://courpharma.com>) has developed negatively charged poly(d,l-lactic-co-glycolic acid) (PLGA) NPs encapsulating disease-specific autoantigens [280]. These tolerogenic NPs, administered intravenously, bind to circulating monocytes, which subsequently migrate to the liver or the spleen and are taken up by resident M ϕ via the macrophage receptor with collagenous structure (MARCO). This process induces immune tolerance by downregulating co-stimulatory markers such as CD80 and CD86, reducing the production of proinflammatory cytokines like IL-12, and upregulating PD-1 expression in M ϕ . The delivered autoantigen is then presented to T cells in a tolerogenic manner, leading to higher expression of anergy markers on T cells and also the absence of CD127, which correlates with FoxP3 expression [281,282]. Clinical trials using these NPs have reached phase 2 for diseases such as celiac disease and primary biliary cholangitis, with preclinical studies underway for MS and T1D [283,284]. Another innovative approach comes from Allero Therapeutics (<https://www.allerotherapeutics.com>), which has developed polyethylene glycol-PLGA NPs that administer autoantigens alongside *Lactococcus lactis* tolerogenic bacterial particles. These NPs are delivered via an innovative mucoadhesive films designed for oromucosal administration. This strategy promotes the phagocytosis of the nanocarriers by submucosal APCs, leading to the expansion of IL-10 producing Tregs [285,286]. While primarily focused on allergen tolerization, this technology is in preclinical development for celiac disease.

Metallic nanocore-based NPs represent another promising variation of these polymeric platforms. TOPAS (<https://topas-therapeutics.com>) has developed antigen-coupled polymeric NP with an iron oxide core [287]. These superparamagnetic small sized NPs efficiently target liver sinusoidal endothelial cells (LSECs), which specialize in the selective uptake of small molecules. Leveraging the liver's tolerogenic environment, these NPs induce the antigen-specific expansion of CD4⁺ Treg FOXP3⁺ cells via TGF- β signalling [288]. These Tregs suppress autoimmune responses in a spleen-independent manner, as demonstrated in an EAE mouse model

of MS [289]. Topas Therapeutics is currently conducting phase 2 clinical trials for celiac disease and pemphigus vulgaris while progressing preclinical studies for T1D and MS. A different approach has been developed by Parvus therapeutics (<https://parvustx.com>), which has devised a platform using iron oxide core NPs encapsulated with a polymer coating and conjugated to a large density array of peptide:MHC complexes. These systemically delivered NPs directly bind to the TCR of autoreactive T cells in the absence of co-stimulatory signals. By targeting CD4⁺ T cells with peptide:MHC class II complexes, the NPs convert triggers the generation and expansion of antigen-specific Tr1 cells, which suppress autoimmune responses in affected tissues of different clinical mouse models. Furthermore, this therapy also promotes the differentiation of cognate B cells into disease-suppressing regulatory B cells, further enhancing local immune tolerance [290–292]. Its recent milestone, resulting from a collaboration with AbbVie (<https://www.abbvie.com>), comprises the clinical trial for inflammatory bowel disease (IBD). Despite the advancements, metallic NPs present certain challenges. Their surface properties can lead to aggregation during production, complicating cargo loading and scaling up manufacturing processes. Additionally, their compact structure and rigidity shed concerns regarding their biodistribution and efficient clearance [293]. Moreover, their limited biodegradability raises concerns about potential accumulation in specific tissues, necessitating careful evaluation and optimization to ensure long-term safety [294].

Finally, **lipid-based NPs** have emerged as a versatile tool in different areas including cosmetics, cancer treatments or mRNA vaccines. Their physicochemical properties can be finely tuned during production, enabling them to elicit either immunogenic or non-immunogenic responses. In the context of immune tolerance, lipid-based NPs offer unique advantages in targeting and modulating immune responses, presenting exciting possibilities for the treatment of autoimmune diseases. One innovative example comes from EVOQ Therapeutics (<https://www.evoqtherapeutics.com>), which has recently developed synthetic HDL nanodiscs composed of a polipoprotein-A1-mimetic peptide 22A and phospholipids conjugated to disease-specific antigens. This platform, initially proven effective as a cancer vaccine for inducing antigen-specific immune responses, has been adapted for autoimmune conditions [295,296]. Following subcutaneous administration, the nanodiscs are directed to lymph nodes, where they are phagocytosed by DCs, which are able to process them and induce tolerance by increasing antigen-specific Tregs and Tr1 cells, reducing inflammatory cytokines, enhancing IL-10 production and downregulating pathogenic IgG titers. Currently in preclinical development, this strategy holds potential for treating various

autoimmune diseases, including T1D, celiac disease, MG, NMO, RA and SLE. Integrated Therapeutics (<https://integratedntx.com>) is developing a lipid NP platform that co-delivers antigens with an immunosuppressive drug. Using a pegylated (PEG) lipid NP formulation, this approach improves biodistribution and cellular uptake by incorporating polyethylene glycol polymers. Furthermore, the antigen can be customized for a specific autoimmune disease and can be delivered as a multi-cargo like protein, peptide, DNA and/or mRNA [297,298]. This strategy has been shown to suppress proinflammatory cytokines such as KC-GRO, TNF α , IL-1 β , and IL-6, leading to the selective expansion of antigen-specific Tregs and Tr1 cells [299]. Though still in preclinical stages, this platform is being explored for potential applications in T1D and MS. Selecta Biosciences adopted a similar approach with PEG lipid NPs containing autoantigens and agents targeting the mammalian target of rapamycin (mTOR) signalling pathway, a key regulator of cell growth and metabolism [300]. Upon IV administration, these NPs localized to the spleen and liver, where they were taken up by DCs, rendering them tolerogenic. This promoted *in vivo* Treg expansion while suppressing antigen-specific T and B cell responses [301,302]. Currently, the company has merged with Cartesian Therapeutics (<https://www.cartesiantherapeutics.com>) to further develop strategies, such as autologous genetically modified CAR T cells against B cell maturation antigen (BCMA) expressed on plasma cells to hunt down pathogenic autoantibodies-producing plasma cells in MG disease [303]. AntolRx (<https://antolrx.com>) employs a novel approach which involves the co-delivery of PEG liposomes encapsulating autoantigens and the tolerogenic aryl hydrocarbon receptor (AhR) ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) [304]. This approach promotes tolerogenic DCs via AhR-dependent induction of SOCS2, inhibiting nuclear factor κ B (NF- κ B) activation and proinflammatory cytokine production. The resulting antigen-specific tolerance leads to Treg expansion, as demonstrated in preclinical T1D models [305]. In addition to T1D, this platform is being developed for MS, RA, psoriasis, and celiac disease. Finally, Dendright, a company which entered into collaboration with Janssen Biotech (<https://www.janssen.com>), designed liposomes incorporating NF- κ B inhibitor 1 α ,25-dihydroxyvitamin D3 (calcitriol) within the lipid bilayer and antigenic peptides in the hydrophilic core. Targeting DCs in lymph nodes, this strategy, promotes tolerance by reducing CD40 expression and upregulating PD-L1 expression on DCs. Preclinical studies have demonstrated antigen-specific tolerance induction in experimental models of RA and T1D [306,307]. Furthermore, phase 1 clinical trials in RA patients have shown a favourable tolerability profile and promising results in tolerance induction. [308].

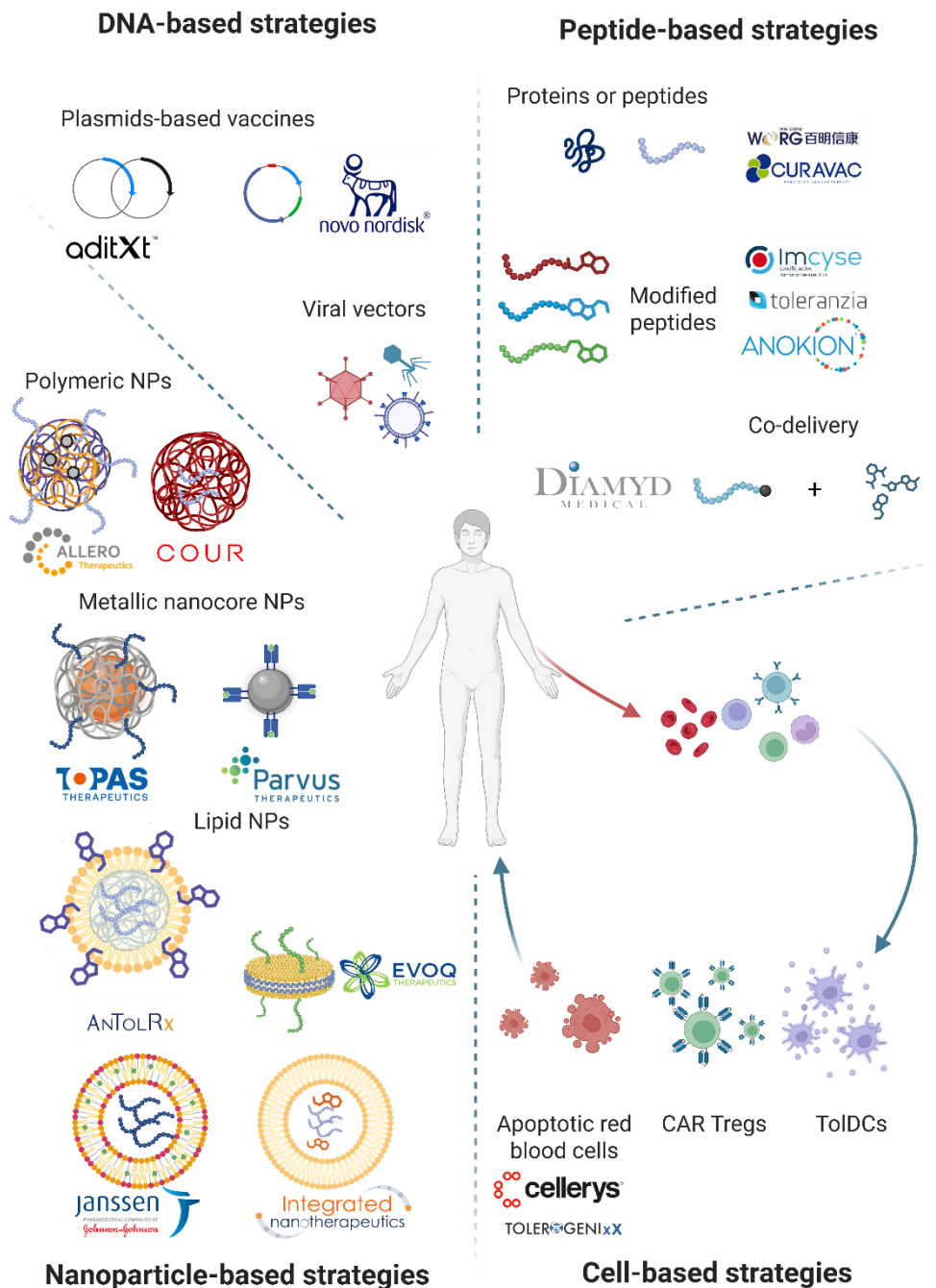


Figure 3. Antigen-specific immunomodulatory therapies for the induction of immune tolerance. DNA-based technologies include plasmid vaccines encoding disease-associated antigens, co-administration with immunomodulators, and multicomponent plasmids, with viral vectors being explored for improved delivery. Peptide-based immunotherapies include strategies like modified peptides, glycopolymer-conjugated autoantigens, and co-delivery with immunomodulators. Cell-based strategies include tolerogenic DCs pulsed with autoantigens, antigen-loaded red blood cells exploiting non-immunogenic clearance, mitomycin C-treated PBMCs, and CAR-Tregs. Nanoparticle-based immunotherapies leverage polymeric, metallic, and lipid NPs to deliver autoantigens with or without immunomodulators. Created in Biorender.com.

3. Modulation of the antigen-specific immune tolerance through efferocytosis

The human body experiences the programmed death, a process known as apoptosis, of over 100 billion cells daily accounting for approximately 0.4% of the total cellular population in an adult [309]. This process is crucial for maintaining tissue homeostasis, as different tissues exhibit varying rates of cell turnover. For instance, haematopoiesis generates billions of short-lived blood cells daily; gastrointestinal epithelial cells are replaced every 4–5 days; and in the thymus and bone marrow, millions of immature thymocytes and B cells are eliminated during their development. Additionally, adult neurogenesis in the brain produces thousands of new neurons daily, only a fraction of which survive. In circumstances such as infections or acute tissue injuries, the rate of apoptosis surpasses normal levels, requiring efficient removal mechanisms to prevent tissue damage [310]. One of these mechanisms is efferocytosis, a homeostatic process, that unlike secondary necrosis, is believed to generate tolerance signals preventing the activation of the immune system against the content of these cells. Leveraging this process, Ahead Therapeutics SL (<https://aheadtherapeutics.com>) has developed synthetic liposomes to mimic apoptotic cells (AC) and trigger an antigen-specific tolerance induction to restore immune homeostasis in autoimmune settings.

3.1. Efferocytosis as a tolerance induction mechanism

It is well-known that the clearance of AC, process termed efferocytosis, is a vital process for maintaining tissue homeostasis under normal physiological conditions and for restoring homeostasis after injury or disease. Although the tolerance induction is not fully understood, significant progress has been made in identifying how immune and phagocytic cells contribute to this process. Over the past three decades, substantial insights have been gained into how AC are recognized and cleared by phagocytes, how their biology is altered by the AC uptake to avoid inflammatory responses and the role of impaired efferocytosis contributes to disease pathology [310–316].

3.1.1. Programmed cell death process

The programmed cell death, or apoptosis, is important to eliminate cells that are aged, damaged or no longer needed, without triggering immune responses or harming surrounding tissues. In contrast to phagocytosis of bacteria or necrotic cells, the clearance of AC is immunologically silent and does not involve the influx of

inflammatory cells into the healthy tissues or the breakdown of the immune tolerance. However, when this process becomes impaired, such as in chronic inflammatory diseases, dead cells accumulate and undergo secondary necrosis, releasing pro-inflammatory cellular contents that can drive autoimmunity, tissue damage, and chronic inflammation [317,318].

Before their recognition by phagocytes, AC undergo distinct morphological changes to facilitate their recognition and further clearance (**Figure 4**). The initial step, for attached cells, involves detachment from surrounding environments, such as extracellular matrix or cell-cell adhesions, a process driven by caspase-mediated cleavage of adhesion components [319–321]. Then occurs plasma membrane blebbing, as a common morphological feature of apoptosis, required for the generation of smaller apoptotic fragments known as apoptotic bodies, which may facilitate their clearance. This process is regulated by caspase-3-mediated activation of RHO-associated kinase 1 (ROCK1), which enhances actomyosin contraction and membrane blebbing [322,323]. AC also release **“find-me” signals** to attract phagocytes. These include soluble molecules like ATP and UTP nucleotides, which recruit monocytes, DCs, and Mφ [324]. Additional signals, like the lipids sphingosine 1-phosphate (S1P) and lysophosphatidylcholine (LPC) or intracellular adhesion molecule 3 (ICAM3) and proteolytically processed CX₃C-chemokine ligand 1 (CX₃CL1), which are packaged into membrane microparticles to enhance monocyte and macrophage chemotaxis [325–329]. Interestingly, some AC simultaneously release factors, referred as a **“keep-out” signals**, such as lactoferrin, to exclude inflammatory cells like neutrophils or eosinophils from the clearance process [330]. When phagocytes are recruited to the proximities, the recognition is achieved by the interaction between ligands present on the AC surface and the corresponding receptors present in the phagocytes. AC expose different ligands on their surface that act as **“eat-me” signals**. Some of these markers are oxidized LDL, calreticulin, C1q and Annexin I, however, the most well-known hallmark of apoptosis is the PS [331]. In healthy cells, PS is confined to the inner leaflet of the membrane by the ATP-dependent flippase ATP11, whereas phosphatidylcholine and sphingomyelin are restricted to the outer leaflet. During apoptosis, caspase 3 cleavages and inactivates ATP11 function and activates scramblase XK-related protein (XKR8), exposing PS on the surface of AC as an **“eat-me” signal** for phagocytes [332,333]. However, apparently displaying certain eat-me signals alone may not be sufficient to trigger AC-engulfment. Dysregulated but healthy cells, like lymphoma cells, can also display PS on their membrane, however, Mφ or DCs do not phagocyte them. These cells are protected by the presence of **“do not eat-me” signals**, such as CD31, CD46 and CD47

[334–336]. Therefore, effective AC clearance requires a sufficient presence of "eat-me" signals combined with the loss of inhibitory signals, enabling recognition and removal by phagocytes.

3.1.2. Phosphatidylserine-driven efferocytosis

Despite the continuous turnover of AC, these cells are rarely observed in healthy physiological conditions, even in tissues with high rates of programmed cell death. This phenomenon underscores the efficiency of efferocytosis, the process responsible for their removal, which operates as a highly efficient mechanism under steady-state conditions. Professional phagocytes, including M ϕ and DCs, play a central role in AC clearance. However, non-professional phagocytes, such as fibroblasts, endothelial cells, and epithelial cells, can also participate in this process, although it is not their primary function [310,337–339].

Research using generic, biochemical and imaging techniques has revealed that efferocytosis is distinct from classical phagocytosis in both morphology and mechanism [311]. This process relies on the phagocyte's ability to express receptors that recognize AC-associated ligands, the reorganization of the phagocyte cytoskeleton to engulf cell-bound AC and the induction of phagosome-lysosome fusion to degrade the cargo [340]. Several AC recognition mechanisms have been identified being the PS the most important AC-associated signal. PS can be recognized directly by specific **cell surface receptors** present in the phagocytes, such as T cell immunoglobulin mucin receptor (TIM)-1, TIM-3 and TIM-4, the CD300 protein family, stabilin-1 and stabilin-2, brain specific angiogenesis inhibitor 1 (BAI1) and the receptor for advanced glycation end-products (RAGE) [341–347]. Alternatively, soluble bridging proteins can mediate the indirect binding of phagocyte surface receptors to PS, such as integrin $\alpha\beta$ 3/5 binding to milk fat globule-EGF factor 8 (MFG-E8), TAM receptors (for example, MERTK, which is bridged by dimers of protein S and GAS6) or CD36 which can be bind to PS either directly or through oxidized LDL [348–350].

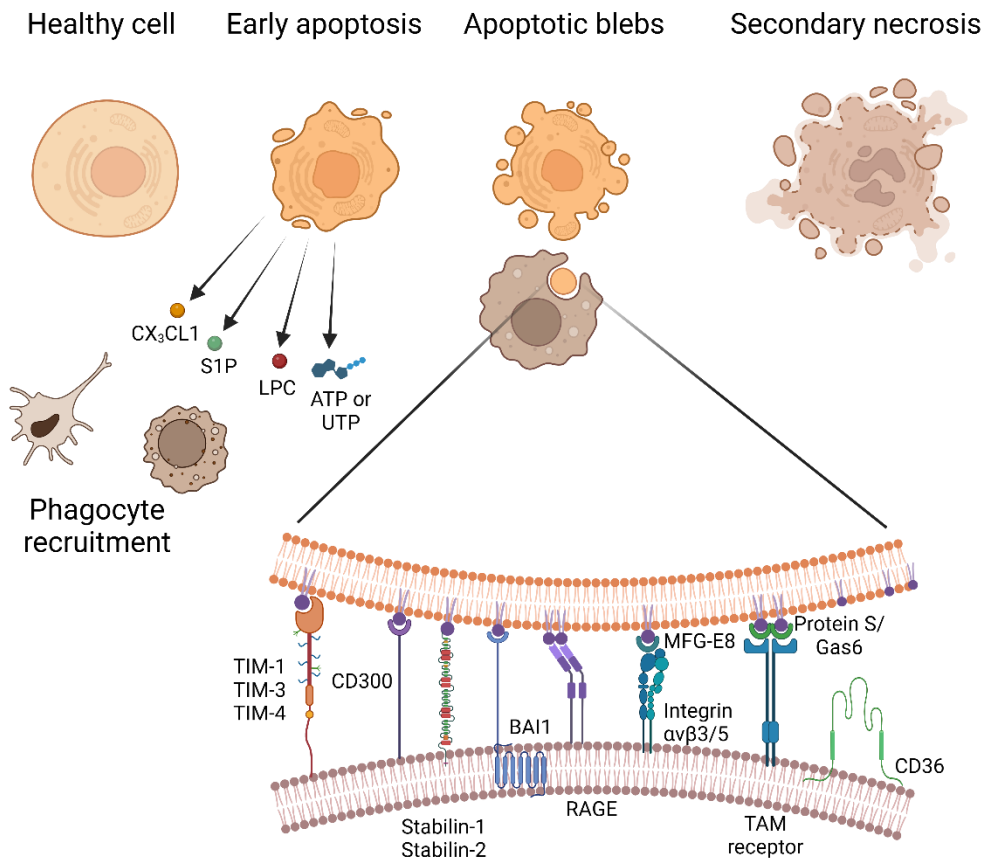


Figure 4. Molecular steps during efferocytosis. First apoptotic cells start releasing chemoattractants such as CX₃CL, S1P, LPC or nucleotides such as ATP or UPT, for the phagocyte recruitment. Next, the recruited phagocytes bind directly or indirectly to the PS (shown as a purple lipid) exposed in the surface of apoptotic cells through receptors, such as TIM-1, TIM-3, TIM-4, the CD300 family, stabilin-1, stabilin-2, BAI1, RAGE, Integrin αvβ3/5, TAM receptors or CD36. Created in Biorender.com.

Engagement of these receptors with PS triggers **downstream signalling pathways**, leading to activation of the protein Rac1. This induces cytoskeletal rearrangements through actin polymerization, enabling the engulfment of AC into phagosome [351]. The phagosome then fuses with lysosomes, where the acidic environment and digestive enzymes degrade the AC components [352]. Since efferocytosis involves engulfing an entire cell, phagocytes must undergo metabolic reprogramming to manage the additional intracellular content, initiating what is known as a homeostatic program [353]. For instance, it has been seen an enhanced cholesterol efflux to regulate lipid balance, a process mediated by the transcription factors of the

PPAR and LXR families. These lipid-sensing pathways not only regulate lipid content but also suppress inflammatory responses, providing a mechanistic distinction between the processing of apoptotic and necrotic cells [354–356]. Furthermore, reprogramming the energy metabolism influences the anti-inflammatory response as well. The engulfment of AC and their processing promotes an anti-inflammatory response mediated by the secretion of IL-10, TGF- β and prostaglandins, and the active inhibition of the production of proinflammatory cytokines, including TNF and IL-1 β [357–361].

3.1.3. Homeostatic maturation in DCs

Efferocytosis, beyond its primary role in preventing secondary necrosis through the efficient clearance of ACs and avoiding the release of intracellular contents that could disrupt immune homeostasis, has several additional critical functions that contribute to maintaining immune balance. A key function is the termination of inflammatory responses. For instance, after immune cells, such as neutrophils or other phagocytes, eliminate extracellular pathogens, efferocytosis ensures their timely removal, preventing prolonged or excessive inflammation that could result in tissue damage. Additionally, efferocytosis plays an essential role in promoting self-tolerance by recognizing and preserving the body's own tissues, thereby preventing autoimmune responses. Furthermore, this process activates pro-resolving pathways to resolve inflammation and avoid unnecessary immune activation through the induction of a tolerogenic phenotype in phagocytic cells to prompt its anti-inflammatory and immunoregulatory effects.

An emerging concept in this context is the “homeostatic maturation” of DCs. Under pathogenic conditions, DCs rapidly undergo classical maturation, marked by functional and morphological changes such as the upregulation of co-stimulatory molecules (CD80, CD86, and CD40), the expression of chemokine receptor CCR7, and the secretion of pro-inflammatory cytokines and chemokines to shape T cell activation. However, DCs can also mature under steady-state or germ-free conditions through a distinct process termed homeostatic or tolerogenic maturation. This form of maturation is crucial for establishing **peripheral tolerance** by presenting self-antigens to autoreactive T cells that escape central tolerance, leading to T cell anergy, deletion, or conversion into Tregs [362]. Notably, homeostatic maturation of thymic DCs has also been shown to be mandatory for central tolerance induction [44]. Despite its importance, the upstream signals and intrinsic regulators of homeostatic DCs maturation have long remained poorly understood. Moreover, it is still debated

whether tolerogenic DCs undergo a full maturation process in homeostatic conditions or induce tolerance due to a semi-mature phenotype.

The **conventional DCs type 1 (cDCs1)** have been described as especially susceptible to undergo this homeostatic maturation, compared to conventional DCs type 2 (cDCs2). cDCs1 are typically found in strategic areas for accessing antigens such as the red pulp and marginal zone of the spleen, in the airways of the lungs or in the villi from the small intestine. Several recent reports indicate that the engulfment of ACs is the key for onset of the homeostatic cDC1s maturation programme and surface exposure of CCR7 [363]. Once they start the maturation, their phagocytic capacity starts to decline, and expression of chemokine receptors starts to promote their migration from peripheral tissues towards the LN, or in the case of resident LN cDCs1, from the medulla to the paracortex. Meanwhile, the antigen processing and presentation machinery becomes upregulated to become professional APCs expressing the homeostatic gene set including *Scube3*, *Eno3* and *Slco5a1* [44,363]. The expression of these genes, for example *Scube3* is linked to the TGF- β signalling pathway, allowing DCs to induce tolerance to self-antigens actively, converting CD4⁺ T cells into Tregs [364]. Of note, the recognition of autoantigens by Tregs leads to the downregulation of the co-stimulatory molecules such as CD80 and CD86 on homeostatically matured DCs through CTLA-4 mediated interactions as a mechanism to prevent naïve T cell stimulation [362]. Finally, these cells can upregulate FAS receptor, a mechanism well-known to prevent autoimmunity [365]. In agreement with this, it has been demonstrated that DCs that have engulfed ACs in tumours or in the intestine, mature and are enriched in the expression of immunoregulatory molecules [366,367]. This tolerogenic mechanism has been related with both central and peripheral tolerance. In the thymus, cDCs1 acquire mTEC-derived self-antigens, mature in a homeostatic manner and present the antigens to the developing T cells [368]. In the periphery it has been demonstrated that DCs, mostly CD103⁺ cDC1s, acquire ACs during efferocytosis and transport it to draining lymph nodes to induce FoxP3⁺ Tregs in lungs, pancreas, spleen and intestine by the production of immunomodulatory molecules such as retinoic acid or TGF- β [362,369–371].

Although cDC1s are the cell population most frequently associated with homeostatic maturation, **other immune cell subsets**, such as cDC2s and M ϕ , have also been shown to undergo tolerogenic maturation following the efferocytosis of ACs [372,373]. This broader susceptibility highlights the potential of AC-based approaches to direct the tolerogenic maturation of multiple immune cell types. Such strategies offer a promising avenue for developing novel therapies aimed at inducing

antigen-specific tolerance, thereby addressing the underlying immune dysregulation in autoimmune diseases and other immune-mediated disorders.

Additional immune cell subsets, such as **B cells**, have also been implicated in the recognition and interaction with ACs. While B cells are traditionally not considered professional phagocytes, accumulating evidence suggests they may participate in the clearance of apoptotic material, primarily through the secretion of the immunoregulatory cytokine IL-10, thereby contributing to the maintenance of immune homeostasis [374]. The mechanisms by which B cells engage with ACs remain incompletely defined. Some studies propose that BCR- or TLR-9-mediated recognition underlies this interaction, whereas others advocate for PS specific receptor-mediated interaction, as TIM-1 [375–377]. Moreover, specific B cell subsets, as splenic B1a cells, have been shown to possess phagocytic capabilities, reinforcing the idea that B cells may play a more active role in efferocytosis than previously appreciated [378].

3.2. Efferocytosis biomimicry to tackle autoimmune settings

The immunomodulatory property of AC makes them as a promising candidate for therapeutics interventions. For instance, extracorporeal photopheresis, a treatment that induces leukocyte apoptosis *ex vivo* before their reinfusion into the body, has demonstrated efficacy in managing transplant rejection and graft-versus-host disease [379–381]. In the field of autoimmune disorders, transfusion of apoptotic NIT-1 cells, an insulinoma cell line, has been shown to halt T1D progression NOD mice, the spontaneous T1D experimental model. This effect is mediated by the suppression of anti- β -cell antibody development and the induction of Th2 responses and IL-10 producing Tr1 cells [382]. Additionally, DCs from NOD mice that engulfed apoptotic β cells, exhibited a tolerogenic phenotype characterized by reduced expression of co-stimulatory molecules (CD40 and CD86), diminished pro-inflammatory cytokine production, and impaired capacity to stimulate autologous T cell proliferation. Transferring these tolerogenic DCs into NOD mice prevented β -cell destruction, decreased T1D incidence, and mitigated lymphocytic infiltration in pancreatic islets [383,384]. However, translating these findings to human applications faced substantial challenges, particularly the limited availability of autologous apoptotic β -cells required for the therapy.

To address this limitation, **antigen-loaded phosphatidylserine (PS)-enriched liposomes** encapsulating insulin peptides were designed to mimic AC by modulating key physicochemical properties (**Figure 5**). First, by enriching liposomes with PS, the

design specifically targets professional APCs involved in AC clearance, thereby promoting self-tolerance by the mechanisms previously mentioned. Furthermore, the inclusion of autoantigens ensures that the immune response is modulated in an antigen-specific manner, without compromising broader immune functions. The surface charge, or ζ -potential, plays a significant role in cellular uptake and immune modulation. Cationic liposomes are known to exhibit adjuvant properties by inducing Th1 immune responses, upregulating co-stimulatory molecules, and promoting pro-inflammatory cytokine production in phagocytic cells, making them suitable for vaccines or cancer therapies. On the other hand, anionic liposomes with a negative charge below -30 mV demonstrate anti-inflammatory properties, as they are preferentially taken up by specialized APCs, promoting antigen-specific immune tolerance [385]. The size and shape of the NPs also influence their interaction with immune cells and drug release kinetics. Larger particles (>500 nm) are primarily internalized through phagocytosis or receptor-mediated endocytosis, often eliciting immune responses, while smaller particles (<50 nm) are taken up by pinocytosis, favouring non-phagocytic cells [386,387]. Based on this, in 2015, negatively charged PS-liposomes encapsulating autoantigens were demonstrated to be efficiently phagocytosed by murine DCs without affecting cell viability. Following phagocytosis, DCs exhibited a tolerogenic phenotype with reduced expression of MHC class I, MHC class II, CD40, and CD86 molecules. With a diminished ability to stimulate autoreactive T cell proliferation and lower secretion of pro-inflammatory cytokines, such as IL-17A and IFN- γ [388]. Interestingly, when fluorescent-labelled insulin-loaded PS-liposomes were administered to prediabetic NOD mice, they reached the pancreas, and the signal observed in draining lymph nodes suggested the migratory potential of engulfing DCs. Furthermore, they demonstrated to arrest autoimmune aggression to β -cells, thus preventing and reducing T1D incidence and the severity of insulinitis while empty PS-liposomes did not replicate these results. Hence, demonstrating the powerful tool of the apoptotic mimicry for the tolerance re-establishment prompted by PS-liposomes in an antigen-specific fashion. Beyond T1D, the platform's adaptability was explored with other autoimmune diseases. *In vivo* studies using an EAE mouse model demonstrated that PS-liposomes encapsulating the MOG₃₅₋₅₅ peptide reduced both disease incidence and clinical severity. Mechanistically, the therapy increased LAG3⁺ T cell populations and promoted prostaglandin E2 secretion by DCs, contributing to the observed therapeutic effects [389].

In 2017, this patented technology (WO2015107140) was licensed to **Ahead Therapeutics** (<https://aheadtherapeutics.com>), a spin-off created for the

development of this therapy, marking the beginning of its translational trajectory and yielding promising results. PS-liposomes encapsulating insulin antigens demonstrated their tolerogenic potential *in vitro*, efficiently engulfed by human monocyte-derived DCs from both adult and paediatric T1D patients, as well as healthy controls. These PS-liposomes induced tolerogenic DCs with an immunoregulatory phenotype characterized by modulated expression of key molecules involved in efferocytosis, antigen presentation, and immune regulation. Functionally, these tolerogenic DCs effectively inhibited autoreactive T cell proliferation. Transcriptional profiling further highlighted the upregulation of tolerogenic genes, including TNFAIP3, TNFSF14, TGF β 1, and VEGFA, underscoring the molecular basis of their immunomodulatory effects [390,391].

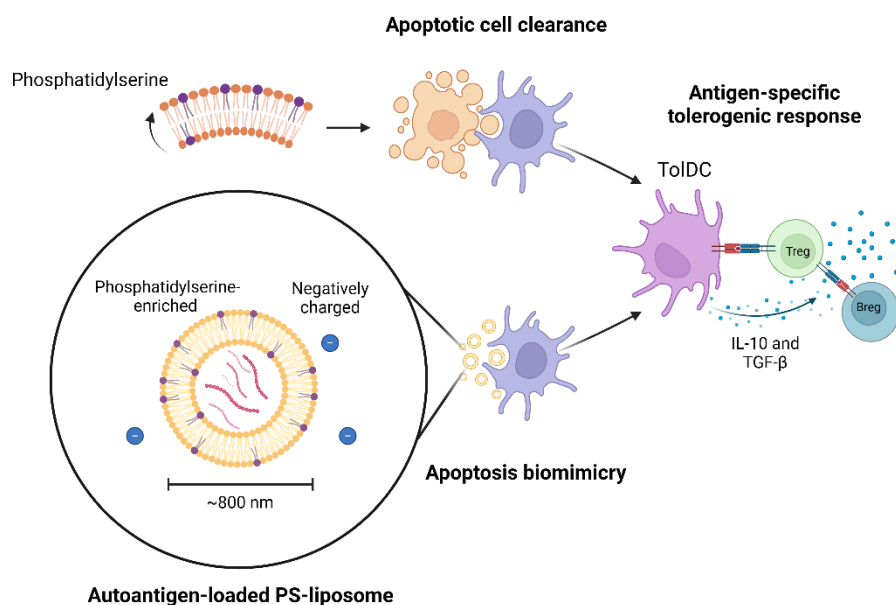
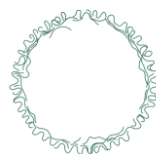
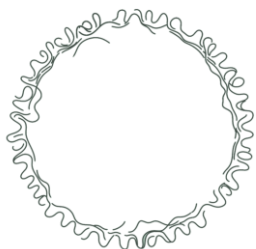
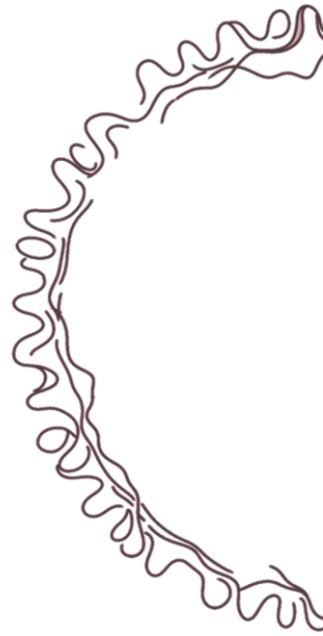


Figure 5. PS-enriched liposome with specific physicochemical characteristics mimics apoptotic cells. PS (shown as purple lipid) is typically expressed on the inner cell membrane of cells, but under a programmed cell death it flips to the outer cell membrane. This serves as a signal for phagocytes to start the apoptotic cell clearance process, also termed as efferocytosis, which through the activation of specific signalling pathways will promote tolerance to all the cellular components of the apoptotic cells. By modulating the physicochemical characteristics of PS-liposomes loaded with autoantigens, the biomimicry of efferocytosis is achieved, in turn, triggering the same tolerogenic pathways and achieving an antigen-specific tolerance induction to autoantigens encapsulated. Created in Biorender.com.

PS-liposomes, by biomimicking the efferocytosis process, offer distinct advantages over other NP-based immunotherapies. They are highly customizable, cost-effective for large-scale production, standardizable, biocompatible, and biodegradable. These features, coupled with their robust immunomodulatory capabilities and adaptability to various autoantigens, highlight their potential as a platform for clinical applications aimed at restoring immune homeostasis in autoimmune diseases. This innovative strategy represents a significant advancement in the pursuit of antigen-specific immunotherapies for autoimmune conditions.



2



HYPOTHESIS AND OBJECTIVES

1. Hypothesis

Although encouraging antigen-specific immunotherapies, that selectively arrest autoimmune aggression have recently been developed, there is still a need for new therapies to restore self-tolerance safely and permanently. In this context, PS-enriched liposomes encapsulating autoantigens have demonstrated a great potential in this field since their ability to mimic apoptotic bodies. When recognized by APCs and subsequently efferocytosed, they trigger the same tolerogenic pathways as apoptotic bodies thereby promoting tolerance against the encapsulated autoantigen. To date, this immunotherapy has been proven to have a platform potential since autoantigen loaded PS-liposomes demonstrated to be effective arresting experimental autoimmune T1D and experimental EAE. Aiming for translation, it has been observed that human monocyte-derived DCs, both from healthy controls or T1D patients, successfully uptake insulin-loaded PS-liposomes. The expression pattern of molecules of these cells concurred with a tolerogenic phenotype. Furthermore, the uptake of these PS-liposomes impaired DCs ability to stimulate autologous T cell proliferation, confirming their tolerogenic impact on human cells. Based on this knowledge, PS-liposomes immunotherapy has emerged as a promising tool for restoring antigen-specific tolerance in human autoimmune diseases.

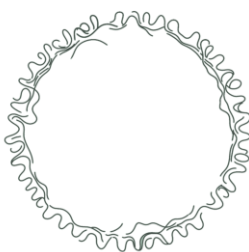
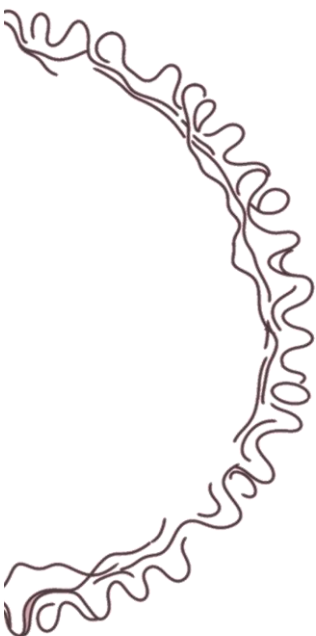
The hypothesis of this work is that PS-enriched liposome-based immunotherapy can safely and efficiently restore antigen-specific immune tolerance in antigen-driven autoimmune diseases. This effect is mediated through the induction of a tolerogenic profile in immune cells that interact with the PS-liposomes, as well as the expansion of regulatory immune cell subsets. Furthermore, the approach is expected to show favourable biocompatibility and safety profiles, supporting its translational potential and laying the groundwork for the future clinical trial.

2. Objectives

The main objective of this work is to gain an in-depth understanding of how the immunotherapy works to design the best approach to ensure efficacy and safety in the future clinical trial.

In order to achieve the main objective, the following specific objectives were defined:

1. To determine the biodistribution of PS-liposomes injected by different administration routes as well as the *in vivo* capture of PS-liposomes by the immune system cells.
2. To characterise the mechanism of action of PS-liposomes and more precisely identify the immune cell subsets that can interact with them.
3. To determine efficacy of PS-liposomes by means of *ex vivo* assays using patient-derived immune cells.
4. To assess the impact of PS-liposomes after exposure to human blood.
5. To develop a potency assay based on the mechanism of action of PS-liposomes ready for its implementation.



3



MATERIALS AND METHODS

1. Phosphatidylserine-liposome production

PS-enriched liposomes (PS-liposomes) were formulated and generated to mimic ACs, as previously described [388–390], and safeguarded under intellectual property rights (WO2015107140A1 and WO2024110516A1 patents). These PS-liposomes comprised a lipid bilayer and encapsulated a variety of autoantigens, thus tailoring loaded PS-liposomes to study specific tolerance induction within each specific autoimmune disorder. Finally, each PS-liposome batch underwent comprehensive characterization to verify their diameter, surface charge, and efficiency in encapsulating peptides.

1.1. Phosphatidylserine-liposomes manufacturing

PS-liposomes were manufactured in Ahead Therapeutic's cleanroom (l'Arboç, Spain) under GMP-like conditions. PS-liposomes were composed of 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS, Lipoid GmbH, Ludwigshafen, Germany), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Lipoid), and cholesterol (CH, Merck, Germany). PS-liposomes were generated from the lipid mixture comprising DOPS, DMPC and CH to mimic apoptotic bodies (molar ratio safeguarded as industrial secret) which were dissolved in ether at 55°C and injected in a solution of phosphate-buffered saline (PBS, Hyclone, GE Healthcare Life Sciences, Marlborough, MA, USA). Liposome emulsion was extruded through a polycarbonate membrane of 1µm pore size using a Lipex Thermobarrel Extruder (Evonik, Burlington, ON, Canada) and dialysed for 24 h. Fluorescent PS-liposomes were prepared as mentioned above by adding 0.15% of 3-hexanoyl-Nitrobenzoxadiazole (NBD) Cholesterol (Cayman Chemical, USA) or Alexa Fluor 750 dye (AF750, ThermoFisher Scientific, Waltham, MA, USA) attached to 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). In the case of loaded PS-liposomes were generated in a PBS solution with 0.5mg/mL of the corresponding peptide to be loaded. Detailed data on the liposome's nomenclature, peptide content, and use are shown in **Table 2**.

Table 2. PS-liposomes used in the study.

Nomenclature	EPP	Species	Use
DOPE-AF750-PS-AChR-liposomes	AChR	Mice	To study the biodistribution of PS-loaded-liposomes in C57 mice
DOPE-AF750-PS-Ins-liposomes	Ins	Mice	To study the biodistribution of PS-loaded-liposomes in NOD mice
DOPE-AF750-PS-liposomes	None	Mice	To control the biodistribution of the autoantigen within the PS-loaded-liposomes
NBD-PS-AChR(h)-liposomes	AChR	Mice and human	To determine interactions between PS-AChR-liposomes and immune cell subsets
NBD-PSIns(h)-liposomes	Ins	Mice	To determine interactions between PS-Ins-liposomes and immune cell subsets
NBD-PS-liposomes	None	Mice and human	To control the effect of the autoantigen within the PS-loaded-liposomes interaction with immune cells
pHrodo-PS-AChR-liposomes	AChR	Human	To evaluate the phagocytosis of PS- liposomes by different immune cell subsets
PS2.5mi-liposomes	2.5mi	Mice	To assess the antigen-specific effect on transgenic NOD mice [392]
PS-AChR-liposomes	AChR	Mice and human	To study the tolerogenic effect of PS-loaded-liposomes on DCs from MG patients
PS-MCV-liposomes	MCV	Human	To study the tolerogenic effect of PS-loaded-liposomes on DCs from AR patients (negative control)
PS-liposome	None	Mice and human	To control the effect of the autoantigen within the PS-loaded-liposomes
PS-SPACER-liposomes	SPACER	Human	To study the peptide HLA presentation in DCs after the PS-loaded-liposome engulfment

2.5mi: 2.5 peptide mimotope; AChR: acetylcholine receptor; EPP: Encapsulated peptide protein; MCV: mutated citrullinated vimentin; Ins: insulin; NOD; non obese diabetic; SPACER; spacer domine of ADAMTS13 protein

1.2. Encapsulated peptides selection

Antigens were selected based on their known relevance to the development of each autoimmune disease. To select the most suitable peptide to encapsulate, a predictive analysis with the informatic tool NetMHCIIpan was performed [393]. The prediction of peptide binding to HLA class II molecules was done by using Artificial Neural Networks (ANNs) and covering the three human MHC class II isotypes HLA-DR, HLA-DQ, and HLA-DP. The final selected peptide sequence of every protein is safeguarded as industrial secret.

Once the peptides were selected –murine 2.5mi and human citrullinated mutated vimentin (MCV), human acetylcholine receptor (AChR) and SPACER domain from ADAMTS13 peptide (SPACER)– those were purchased from Peptide Synthesis Facility (Department of Experimental and Health Sciences, Pompeu Fabra University, Spain) with >95% purity; human insulin was purchased from Merck (Darmstadt, Germany). They were reconstituted using PBS (Hyclone) to 0.5 mg/ml, except for MCV, which was resuspended to 1 mg/ml to ensure optimal encapsulation.

1.3. PS-liposomes characterisation

The particle diameter — expressed as hydrodynamic size—, the distribution of the sizes — polydispersity index (Pdl)— and the surface charge — zeta potential (ζ)— were assessed in undiluted samples of each PS-liposomes batch by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK). Peptide encapsulation efficiencies (EE) were calculated indirectly via the equation:

$$EE (\%) = \frac{C_{total\ peptide} - C_{free\ peptide}}{C_{total\ peptide}} \times 100$$

Total peptide concentration refers to the initial amount of peptide, while the free peptide is the concentration of non-encapsulated peptide. To quantify the free peptide, PS-liposome emulsions were centrifuged at 3,700g at room temperature (RT) for 20 minutes. The concentration of non-encapsulated peptide in the supernatants was measured using the PIERCE BCA protein assay kit (ThermoFisher Scientific). This method is based on the reduction of Cu^{2+} to Cu^{1+} in an alkaline medium, followed by a colorimetric reaction between Cu^{1+} and bicinchoninic acid, which correlates linearly with protein concentration. The tests were carried out in 96-well plates (NUC Maxisorp 96-well ELISA plate, ThermoFisher Scientific), with standard curves generated using the same peptide being evaluated for

encapsulation. Absorbance was read at 562 nm using the Varioskan® spectrophotometer (ThermoFisher Scientific) after 1 hour of incubation at 37 °C, or 2 hours for supernatants containing 2.5mi peptide.

1.4. PHrodo staining

The amino-reactive pHrodo™ iFL Green dye (ThermoFisher Scientific) is an innovative fluorogenic dye, which demonstrates a substantial increase in fluorescence intensity correlated with increasing acidity of its environmental pH. It has excitation and emission of approximately 505 nm and 525 nm, respectively, and can be detectable using standard FITC (fluorescein) or Alexa Fluor™ 488 filters. Furthermore, it can be conjugated to biomolecules, as it readily reacts with the primary amines of proteins, forming a covalently attached fluorogenic pH probe which exhibits high fluorescence exclusively in acidic environments, such as those found in cell lysosomes and endosomes. Given the presence of an amino group on the polar head of DOPS, the staining of PS-liposomes provides an avenue for conducting cell internalization studies characterized by a low signal-to-background ratio and heightened specificity.

For the staining of PS-liposomes, the amino-reactive pHrodo™ iFL Green dye (ThermoFisher Scientific) was appropriately diluted in dimethyl sulfoxide (DMSO (Sigma) following manufacturer's guidelines. The reaction buffer was meticulously prepared with 0.1M sodium bicarbonate (NaHCO₃) and distilled water to achieve a final pH of 8.3. The slightly basic pH was selected to maintain the amine group in a non-protonated form, as amine-reactive agents do not react with protonated aliphatic amine groups. Once the buffer was prepared, the PS-liposomes were diluted at a final concentration of 1 mg/mL and were slowly mixed with the proper amount of pHrodo™ iFL Green dye stock solution depending on the desired molar ratio (dye: DOPS). Subsequently, the mixture was incubated at RT for 60 minutes in the dark under gentle agitation conditions to ensure uniform conjugation.

Following the incubation period, to eliminate any free dye that might not be bound to the PS-liposomes, the mixture underwent dialysis utilizing 1 mL Float-A-Lyzer® Devices (Repligen, Waltham, MA, USA) with a cutoff point of 100 kDa. This implies that only molecules with a size smaller than the specified cutoff point could diffuse across the membrane, as the free pHrodo dye with a molecular weight of 1000Da. Prior to the dialysis process, the devices were pre-hydrated following the manufacturer's guidelines. Subsequently, they were filled with the dye-liposome mixture and then immersed in PBS (Oxoid Limited, Hampshire, UK) for 24h

maintaining a volume ratio of 1:10. To ensure the absence of free dye in the media, the PBS was replaced a total of five times during the dialysis process.

Finally, dialysed pHrodo-PS-liposomes were collected. To check the increasing fluorescence of the pHrodo dye with the pH, stained PS-liposomes were incubated with increasing concentrations of hydrochloric acid (HCl) and then the FITC fluorescence was evaluated using flow cytometry (FACS Canto II, BD Biosciences).

2. Experimental models to study autoimmune diseases

Murine models were used to address the first three objectives of this study. Specifically, non-obese diabetic (NOD) and C57BL/6 mouse strains were used to investigate the biodistribution of PS-liposomes via different administration routes and to study *in vivo* interactions between PS-liposomes and immune cell subsets. The NOD.BDC2.5 mouse strain was employed to evaluate the antigen-specific therapeutic efficacy.

2.1. Ethics

All experiments involving animal models were conducted following the rules and regulations of the animal facilities. The study adhered to the recommendations of the Declaration of Helsinki for animal experimental investigation and the Principles of Laboratory Animal Care established by the National Institute of Health. The protocols received approval from the animal ethics committees of the participating institutions' governments. The permit DAAM from Catalan Government for experiments using the animal models of T1D was 10822; the one for experiments using the C57BL/6 mice was 12139.

2.2. NOD mice

Wild-type NOD mice, which spontaneously develop autoimmune diabetes after 12 weeks of age, were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred under specific-pathogen-free (SPF) conditions at the Centre for Comparative Medicine and Bioimage in Badalona, Spain [394]. Mice were accommodated in a facility under controlled temperature (20-23°C) and humidity (34-83%) conditions, provided with *ad libitum* access to both food and water, and maintained on a 12-hour dark/light cycle.

2.3. NOD.BDC2.5 mice

NOD.BDC2.5 mice, which carry the rearranged TCR genes *Tcra* and *Tcrb* from the diabetogenic H2-Ag7 restricted BDC2.5-CD4⁺ T cell clone, were also acquired from the Jackson Laboratory [392,395]. This strain also develops spontaneous autoimmune diabetes, with a 12% incidence rate by 20 weeks of age [396]. Mice were maintained under the same SPF conditions as wild-type NOD mice.

2.4. C57BL/6 mice

C57BL/6 mice, a genetically standardized inbred strain extensively used in immunological and biomedical research, were also obtained from Charles River (Wilmington, MA, USA). Mice were bred and maintained under SPF conditions when it was required or in a standard laboratory environment at the Centre for Comparative Medicine and Bioimage in Badalona, Spain. The animals were always housed in a controlled environment with regulated temperature and humidity, provided with unrestricted access to food and water, and kept on a 12-hour light/dark cycle to ensure consistent circadian rhythms.

3. Human biological samples

To determine the PS-liposome's ability to interact with human immune cells and assess their tolerogenic potential following phagocytosis, monocyte-derived DCs and peripheral blood mononuclear cells (PBMCs) from healthy individuals and patients with MG and RA were examined. Additionally, due to the availability of synovial fluid from some RA patients, synovial fluid mononuclear cells (SFMCs) were also tested with PS-MCV-liposomes to evaluate their tolerogenic effects on these cells.

3.1. Ethics

All the studies involving human cells were conducted following the principles outlined in the Declaration of Helsinki for human research and were approved by the respective Committees on Ethics Research of the participating institutions. The research carried out in the Germans Trias i Pujol research institute were approved by the Committee of the Ethics of Research of the Germans Trias i Pujol Hospital (reference number PI-22-026), for biological samples collected there. The extraction of MG patients' blood was approved by the committee on the Ethics of Santa Creu I Sant Pau Hospital (Barcelona, Spain) with reference number AT-MG-2019-01, and for

RA biological samples, the Ethics Committee of Vall d'Hebron Hospital (Barcelona, Spain) provided the approval (reference number AR_EX_1001). Blood samples from control subjects obtained at Sanquin (Amsterdam, The Netherlands) were approved by the Committee on Ethics of Sanquin Research Centre. All study participants gave informed consent. The collection and processing of personal and clinical data were restricted to the information necessary for achieving the study objectives, and data were handled with adequate precautions to ensure confidentiality and compliance with applicable data privacy laws and regulations.

3.2. Blood samples from control subjects and patients

Samples of peripheral blood were obtained from adult healthy control subjects and individuals diagnosed with MG or RA. Inclusion criteria were universally established, all participants needed to be 18-55 years of age, have a body mass index (BMI) between 18.5 and 30 kg/m². In case of MG patients, all of them presented positivity titers for anti-AChR antibodies, while all RA patients carried HLA-DR4 allele and CCP levels were > 59UA. Conversely, exclusion criteria encompassed concurrent presence of additional autoimmune disorders, pregnancy or breastfeeding, compromised kidney functionality, or prevailing liver conditions.

3.3. Synovial fluid samples from RA patients

RA is characterized by joint inflammation, particularly affecting the synovial membrane, leading to synovitis. This inflammation results in excessive synovial fluid production, causing joint swelling and stretching of the joint capsule [151]. Synovial fluid samples to study were collected from RA patients exhibiting synovial inflammation. The inclusion and exclusion criteria for sample collection were consistent with those previously described.

3.4. Clinical data management

Clinical data such as sex, age and date of diagnosis were collected from all patients, while maintaining patient anonymity by encoding their personal data. In MG patients, anti-AChR titers (nmol/L), clinical manifestation, thymic pathology and the MGFA (Myasthenia Gravis Foundation of America Classification) classes which reflects; pure ocular manifestation of MG (class I), mild generalized manifestation (class II), moderate generalized manifestation (class III), severe generalized manifestation (class IV), and intubation/myasthenic crisis (class V); A indicates a

predominant affection of limb or/and axial muscles while B indicates a predominant affection of oropharyngeal or /and respiratory muscles.

In RA patients, to measure clinical disease activity on a continuous scale a Disease Activity Score 28 (DAS28) was used. This score transforms and weight different component variables, including tender joint count (TJC), swollen joint count (SJC), erythrocyte sedimentation rate (ESR) and global health (GH) (**Table 3**). Clinical data as presence of RF, ACPA and the HLA-DR typing were also collected.

Table 3. DAS28 score equation and disease activity measures.

$DAS28 = (0.56 \times \sqrt{TJC}) + (0.28 \times \sqrt{SJC}) + (0.7 \times \ln ESR) + (0.014 \times GH)$			
≤ 2.6	$>2.6 - 3.2$	$> 3.2 - 5.1$	> 5.1
Remission	Low disease activity	Moderate disease activity	High disease activity

DAS28, Disease activity score 28; ESR, erythrocyte sedimentation rate; GH, global health; SJC, swollen joint count; TJC, tender joint count.

4. PS-liposomes biodistribution

To decide which administration route of PS-liposomes was the most optimal to administrate them, *in vivo* tracking of fluorescently labelled PS-liposomes was performed using different methods of injection. Empty or antigen-loaded DOPE-AF750-labelled PS-liposomes were administered via intraperitoneal (IP), intradermal (ID), IV and intranasal (IN) routes in a single dose of 200µl, 50µl, 100µl and 50 µl respectively, to prediabetic NOD mice (<12 weeks-of-age) or C57BL/6 mice. Animals administered IN received 2 µL of PS-liposomes per nostril alternately until a cumulative volume of 50 µL was reached, employing a pipette for precise delivery. Afterwards PS-liposome administration, *in vivo* imaging was conducted at time points of 15 minutes, 1h, 3h, 6h, 24h and 48h after the administration, either on live mice under isoflurane sedation or following animal euthanasia and organ harvest for subsequent analysis. Near-infrared (NIR, 0.7-1.7µm) fluorescence imaging was performed using the Pearl Impulse imaging system (LI-COR Biosciences, Lincoln, NE, USA) to detect fluorescence in the subsequent organs, including: axillary lymph nodes (axillary LN), bladder, brain, heart, hind limb's bones, kidneys, liver, lungs, mediastinal lymph nodes (MDLN), pancreas, perigonadal adipose tissue (PAT), PLN, salivary gland, spleen, stomach, and thymus, which had been previously harvested

and washed with PBS. To standardize fluorescence values, background fluorescence was subtracted. The resulting fluorescence signal was quantified as relative fluorescence units (RFU) per gram of tissue for each respective organ using the Image Studio 5.5.4 Software (LI-COR Biosciences).

5. Cell viability and count assessment

Viability and cell number assessment was always conducted using 7-Amino-Actinomycin D (7-AAD; BD Biosciences), which was incubated with the cells to assess for 15 minutes at RT. The Accucheck Counting Beads (ThermoFisher Scientific) were added then, and the sample was assessed using flow cytometry (FACS Canto II, BD Biosciences).

6. *In vivo* capture of fluorescent PS-liposomes

To further explore which immune cell subsets were able to interact *in vivo* with the fluorescent PS-liposomes, prediabetic NOD mice were treated with fluorescent-labelled empty NBD-PS-liposomes or insulin-loaded NBD-PS-Ins(h)-liposomes administered IP in a single dose of 200 μ l, ID in a single dose of 50 μ l or IV in a single dose of 100 μ l. CD57BL/6 mice were treated with fluorescent-labelled NBD-PS-AChR-liposomes administered IV in a single dose of 100 μ l. After 15 minutes, 1-, 6-, 24- or 48-hours, blood and organs of interest were collected and placed in their respective appropriate media.

Blood was obtained via intracardiac puncture from mice under terminal anaesthesia induced by an IP administration of 200 μ l of a lethal dose of 75 mg/ml ketamine (KETAMIDOR®, Alvira, Barcelona, Spain) and 10 mg/mL xylazine (Alvira). For extraction, a syringe equipped with a 25-gauge needle was employed, and immediately after the collection, the blood was placed into a tube containing 1.6mg of Ethylenediaminetetraacetic acid (EDTA) (SARSTEDT, Nümbrecht, Germany) and vigorously mixed. After that, blood was transferred into a 15 mL Falcon and centrifuged at 2300 x g for 5 minutes at RT. Subsequently, the plasma fraction was removed, and cold Ammonium-Chloride-Potassium (ACK) isotonic lysis buffer (8.2 g/L Ammonium Chloride (NH₄Cl) (Probus, Badalona, Spain), 1g/L Potassium bicarbonate (KHCO₃) (Sigma, Saint, Louis, MI, USA), 37 mg/L EDTA (Sigma)) was added to the remaining cellular fraction in a 1:10 ratio and then incubated for 10 minutes in dark at 4°C. The samples were washed twice at 400 x g for 5 minutes at RT with 5mL of RPMI-164 media (Biowest) supplemented with 10% foetal bovine

serum (FBS; ThermoFisher Scientific) to stop the lysis process. Finally, cells were resuspended in 1mL of PBS (Oxoid Limited) supplemented with 2% of FBS (ThermoFisher Scientific).

After collecting the spleen, it was maintained in PBS (Oxoid Limited) supplemented with 2% of FBS (ThermoFisher Scientific) until it was gently mechanically disrupted. The resulting volume was filled up until 5mL and the desegregation was sedimented for 2 minutes to remove the conjunctive tissue and debris. The supernatant was collected and washed at 400 x g for 5 minutes at RT. After decanting the supernatant, erythrocytes were haemolysed using 5mL of tempered haemolysis solution (500 mL deionised H₂O (Mili-Q® Direct, Merck Milipore, Burlington, MA, USA), 1.0297 g Trizma Hydrochloride (Sigma) and 3.735 g NH₄Cl (Probus) during 5 minutes at RT. Subsequently, 5 mL of RPMI-164 media (Biowest) supplemented with 10% FBS (ThermoFisher Scientific) were added to stop the lysis process. After washing the samples at 400 x g for 5 minutes at RT, the pellet was resuspended in PBS (Oxoid Limited) supplemented with 2% of FBS (ThermoFisher Scientific).

PLN and MDLN were collected in PBS (Oxoid Limited) supplemented with 2% of FBS (ThermoFisher Scientific) until they were gently mechanically disrupted. The resulting volume was filled up until 5mL and washed at 400 x g for 5 minutes at RT. After removing the supernatant, the pellet was resuspended in PBS (Oxoid Limited) supplemented with 2% of FBS (ThermoFisher Scientific).

The liver was collected, with the gallbladder removed, and then preserved in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific). Subsequently, it was transferred to gentleMACS™ C Tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) containing dissociation mix pre-incubated for 30 minutes at 37°C in DMEM. The single cell suspension was obtained according to the manufacturer's provided instructions (Liver Dissociation Kit mouse, Miltenyi Biotec) using the gentleMACS Dissociator (Miltenyi Biotec). The resulting suspension was filtered using 100 µm filters MACS® SmartStrainer (Miltenyi Biotec) and then washed with DMEM (ThermoFisher Scientific) at 400 x g for 5 minutes at RT. After decanting the supernatant, erythrocytes were haemolysed during 5 minutes at RT using 5 mL of tempered haemolysis solution, composed of 2.059 g/L of Trizma Hydrochloride (Sigma) and 7.47 g/L of NH₄Cl (Probus). Subsequently, 5mL of RPMI-164 media (Biowest) supplemented with 10% FBS (ThermoFisher Scientific) were added to stop the lysis process. After washing the samples at 400 x g for 5 minutes at RT, the pellet

was resuspended in PBS (Oxoid Limited) supplemented with 2% of FBS (ThermoFisher Scientific).

After preparation of all single cell suspensions, the cell count of each sample was determined according to the procedures described in **section 5**. The proper amount of volume of each sample was transferred into an Eppendorf tube and centrifuged at 400 x g for 5 minutes at RT. After supernatant removal, the pellet was resuspended and stained for 20 minutes at 4°C with the following monoclonal antibodies listed in **Table 4**. Fixable Viability Stain 575V (BD Biosciences, USA) staining was used to exclude dead cells. After that, PBS (Oxoid Limited) supplemented with 2% of FBS (ThermoFisher Scientific) was added to every sample and then centrifuged at 10.000 rpm for 30 seconds. Samples were permeabilized and fixed following the manufacturer's provided instructions (True-Nuclear™ Transcription Factor Buffer Set, BioLegend, San Diego, CA, USA) and FoxP3 and CD68 BV605 antibodies were used as intracellular staining. Cells were acquired using flow cytometry (FACS LSR Fortessa, BD Biosciences) and corresponding fluorescence minus one (FMO) staining was used as a control. FlowJo software (Tree Star, OR, USA) was used for the data analysis.

7. Assessment of liposome antigen-specific *in vivo* effect

Antigen-specific effect of PS-liposomes was assessed by treating NOD.BDC2.5 mice either with diabetogenic peptide 2.5mi loaded PS-2.5mi-liposomes or empty PS-liposomes. After 4 or 13 days of a single dose administration of 100 µl IV or a 50 µl dose ID mice were euthanized with an IP administration of 200 µl of a lethal dose (75 mg/mL ketamine and 10 mg/mL xylazine). Spleen and pancreatic lymph nodes were collected as described in **section 6**. After obtaining the single cell suspensions, the cell count of each sample was determined according to the procedures described in **section 5**.

Table 4. List of monoclonal antibodies used to detect the immune cell subsets in NOD and CD57BL/6 mice after the administration of fluorescent-labelled, empty or loaded, NBD-PS-liposomes

Target	Fluorophore	Species/Isotype	Clone	Use *	Company
B220	BV510	Rat IgG2a, κ	RA3-6B2	1:250	BD Biosciences
CD11b	PECy5	Rat IgG2b, κ	M1/70	1:250	BioLegend
CD11c	BV786	Armenian Hamster IgG1, λ2	SHCL-3	1:100	BD Biosciences
CD14	PEDazzle594	Rat IgG2a, κ	Sa14-2	1:250	BioLegend
CD146	PECy7	Rat IgG2a, κ	ME-9F1	1:500	BioLegend
CD19	BV570	Rat IgG2a, κ	6D5	1:250	BioLegend
CD1d	PECy7	Rat IgG2b, κ	1B1	1:500	BioLegend
CD3	PerCP	Armenian Hamster IgG1, κ	145-2C11	1:50	BD Biosciences
CD4	BV711	Rat IgG2b, κ	GK1.5	1:500	BioLegend
CD45	APCFire810	Rat IgG2b, κ	30-F11	1:100	BioLegend
CD49b	PE-CF594	Rat Lewis IgG1, κ	DX5	1:250	BD Biosciences
CD5	BV605	Rat LOU/M IgG2a, κ	53-7.3	1:250	BD Biosciences
CD68	BV605	Rat IgG2a	FA-11	1:250	BioLegend
CD8	BV786	Rat LOU/M IgG2a, κ	53-6.7	1:100	BD Biosciences
CD8a	BV711	Rat IgG2a, κ	53-6.7	1:250	BioLegend
F4/80	APC-R700	Rat Wl IgG2a, κ	T45-2342	1:250	BD Biosciences
Foxp3	PE	Rat / IgG2a, κ	FJK-16s	1:150	Invitrogen
LAG3	APC	Rat Lewis IgG1, κ	C9B7W	1:50	BD Biosciences
Ly6C	APC	Rat IgG2c, κ	HK1.4	1:250	BioLegend
Ly6G	BV607	Rat IgG2a, κ	1A8	1:250	BioLegend
MHC-II I-A[b]	PE	Mouse BALB/c IgG2a, κ	AF6-120.1	1:500	BD Biosciences
MHC-II I-Ad	PE	Mouse SJL IgG2b, κ	AMS-32.1	1:500	BD Biosciences
TIGIT	APC-R700	Mouse IgG1, κ	1G9	1:50	BD Biosciences
TIM-1	BV421	Rat Sprague-Dawley IgG2b	RMT1-4	1:250	BD Biosciences

* Prior to use, each antibody underwent a process of titration.

The proper amount of volume of each sample was transferred into an Eppendorf tube and centrifuged at 400 x g for 5 minutes at RT. After supernatant removal, the pellet was resuspended and stained for 20 minutes at 4°C with the following monoclonal antibodies listed in **Table 5**. Fixable Viability Stain 575V (BD Biosciences, USA) staining was used to exclude dead cells. After that, PBS (Oxoid Limited) supplemented with 2% of FBS (ThermoFisher Scientific) was added to every sample and then centrifuged at 10,000 x g for 30 seconds. Samples were permeabilized, fixed and Foxp3 marker was intracellularly stained following the manufacturer's provided instructions (Anti-Mouse/Rat FoxP3 Staining Set FITC, ThermoFisher Scientific). Cells were acquired using flow cytometry (FACS LSR Fortessa, BD Biosciences) and corresponding FMO staining was used as a control. To check the expression of 2.5mi TCR transgene on CD4⁺ T cells, a test using the tetramer MHC class II: Ag⁷/2.5mi peptide (AHHPIWARMDA) (GenScript Biotech, Piscataway, NJ, USA) was performed. FlowJo software (Tree Star, OR, USA) was used for the data analysis.

8. PS-liposomes effect in human monocyte-derived DCs *in vitro*

As described, DCs act as a critical bridge between the innate and adaptive immune response orchestrating the communication by presenting peptides resulting from antigen processing after phagocytosis [397]. To investigate whether the engulfment of PS-liposomes by human DCs induces tolerance towards the autoantigen encapsulated within the PS-liposomes, blood samples from patients with MG and RA were collected and processed. A schematic workflow of the protocol is represented in **Figure 6**.

Table 5. List of monoclonal antibodies used to detect the immune cell subsets in mice after PS-2.5mi-liposome injection to BDC2.5 mice

Target	Fluorophore	Species/Isotype	Clone	Use *	Company
CD152/ CTLA4	APC	Armenian Hamster IgG1, κ	UC10-4F10-11	1:50	BD Biosciences
CD19	APC-R700	Rat LEW IgG2a, κ	1D3	1:250	BD Biosciences
CD197/ CCR7	PECF594	Rat LOU IgG2a	4B12	1:250	BD Biosciences
CD1d	PECy7	Rat IgG2b, κ	1B1	1:250	BioLegend
CD25	BV421	Rat LEW IgG2a, κ	3C7	1:100	BD Biosciences
CD279/PD-1	BV510	Armenian Hamster IgG2, κ	J43	1:250	BD Biosciences
CD3	PerCP	Armenian Hamster IgG1, κ	145-2C11	1:50	BD Biosciences
CD4	APCCy7	Rat LEW IgG2b, κ	GK1.5	1:100	BD Biosciences
CD44	PECy7	Rat IgG2b, κ	IM7	1:250	BD Biosciences
CD49b	BV421	Rat LEW IgM, κ	DX5	1:50	BD Biosciences
CD62L	BV510	Rat F344 IgG2a, κ	MEL-14	1:250	BD Biosciences
CD69	BV711	Armenian Hamster IgG1, λ3	H1.2F3	1:250	BD Biosciences
CD73	PE/Dazzle594	Rat IgG1, κ	TY/11.8	1:250	BioLegend
CD8a	BV785	Rat LOU IgG2a, κ	53-6.7	1:200	BD Biosciences
Foxp3	FITC	Rat IgG2a, κ	FJK-16S	1:40	ThermoFisher Scientific
FR4	BV650	Rat WI IgG1	12A5	1:250	BD Biosciences
LAG3	APC	Rat LEW IgG1, κ	C9B7W	1:50	BD Biosciences
MHC-II/MHC-I-A[d]	PE	Mouse SJL IgG2b, κ	AMS-32.1	1:500	BD Biosciences
TIGIT	BV711	Mouse IgG1, κ	1G9	1:250	BD Biosciences

* Prior to use, each antibody underwent a process of titration.

8.1. Peripheral Blood Mononuclear Cells obtaining

Blood samples from venepuncture were collected using BD Vacutainer Sodium Heparin tubes (BD Biosciences) until reaching a total volume of 50 mL and subsequently diluted 1:1 with PBS (Oxoid Limited). PBMCs were acquired using Ficoll Paque (GE Healthcare Life Sciences) density gradient centrifugation. The process involved centrifuging at 700 x g without brake for 30 minutes at RT, after adding two volumes of diluted blood to one volume of Ficoll solution carefully ensuring meticulous separation of the two phases. Following centrifugation, the PBMCs layer was aspirated using a Pasteur pipette, followed by the removal of platelets through subsequent centrifugation at 200 x g for 15 minutes at RT. Cells were resuspended in PBS (Oxoid Limited) supplemented with 2% FBS (ThermoFisher Scientific) medium. Viability and cell number assessment was conducted using Annexin V PE staining (Immunotools, Friesoythe, Germany) and 7-AAD (BD Biosciences). The percentage of CD14 positive cells was assessed with the anti-human CD14 PE antibody (Immunotools) followed by an incubation at RT for 15 minutes. The Accucheck Counting Beads (ThermoFisher Scientific) were added, and the sample was assessed using flow cytometry (FACS Canto II, BD Biosciences). For subsequent analyses, a portion of whole PBMCs were cryopreserved in FBS (ThermoFisher Scientific) with 10% DMSO (Sigma) at a concentration of $15\text{--}20 \times 10^6$ cells/mL.

8.2. CD14 positive fraction isolation from PBMCs

The rest of the isolated PBMCs were suspended to a concentration of 100×10^6 cells/mL. The suspension was then transferred to a 5 mL Polystyrene Round-Bottom Tube (BD Biosciences) to facilitate the subsequent magnetic isolation of monocytes using the EasySep Human CD14 Positive Selection Kit (STEMCELL Technologies, Vancouver, Canada). A volume equivalent to one-tenth of the CD14 Positive Selection cocktail was added to the cell suspension, followed by an incubation at RT for 15 minutes. Subsequently, a volume corresponding to a twentieth of the Magnetic Nanoparticles was added and incubated for 10 minutes at RT. To achieve a final volume of 2.5 mL, the cell suspension was adjusted with PBS (Oxoid Limited) supplemented with 2% FBS (ThermoFisher Scientific) medium. The resulting mixture was then positioned within the EasySep magnet (STEMCELL Technologies) for 5 minutes. The volume was poured off and the attached cells were mixed with PBS (Oxoid Limited) with 2% FBS (ThermoFisher Scientific) medium. The magnet isolation process was repeated until the proportion of CD14 positive cells exceeded 70%, confirmed by CD14 PE (Immunotools) and 7-AAD (BD Biosciences) viability staining,

together with the Accuclick Counting Beads (ThermoFisher Scientific) using flow cytometry (FACS Canto II, BD Biosciences) (see **section 5**). Monocytes were cryopreserved in FBS (ThermoFisher Scientific) with 10% DMSO (Sigma) at a concentration of 10×10^6 cells/mL and stored for later use. Viability and cell count assessments were also conducted on the CD14 negative fraction to subsequently cryopreserve the cells at $15\text{--}20 \times 10^6$ cells/mL as previously mentioned.

8.3. Synovial Fluid Mononuclear Cells isolation

The synovial fluid samples were collected using a BD Vacutainer Sodium Heparin tube (BD Biosciences) and subsequently, diluted at a 1:1 ratio in a 50 mL Falcon tube with RPMI-164 media (Biowest, Nuaille, France) supplemented with 100 IU/mL penicillin (Normon SA, Madrid, Spain), 100 µg/mL streptomycin (Laboratorio Reig Jofré, Barcelona, Spain) and 2mM L-glutamine (Sigma-Aldrich). Hyaluronidase (Sigma-Aldrich) was added reaching the final concentration of 10 U/mL by gentle pipetting to proper mixing the sample, followed by a 30 minutes incubation at 37°C. Afterwards, to obtain the SFMCs, the sample was centrifugated at $895 \times g$ without brake for 30 minutes at RT previously having added two volumes of synovial sample to one volume of Ficoll Paque (GE Healthcare Life Sciences). Once the sample was layered, the SFMCs were harvested using a Pasteur pipette and transferred to a new Falcon tube. Cells were washed twice with RPMI-164 media (Biowest) supplemented with 10% FBS (ThermoFisher Scientific), 100 IU/mL penicillin (Normon SA) and 100 µg/mL streptomycin (Laboratorio Reig Jofré). Finally, SFMCs were resuspended in PBS (Oxid Limited) supplemented with 2% FBS (ThermoFisher Scientific) medium. Viability and cell number assessment was conducted as described in **section 5**. Finally, the whole fraction of SFMCs were cryopreserved at $15\text{--}20 \times 10^6$ cells/mL as previously mentioned.

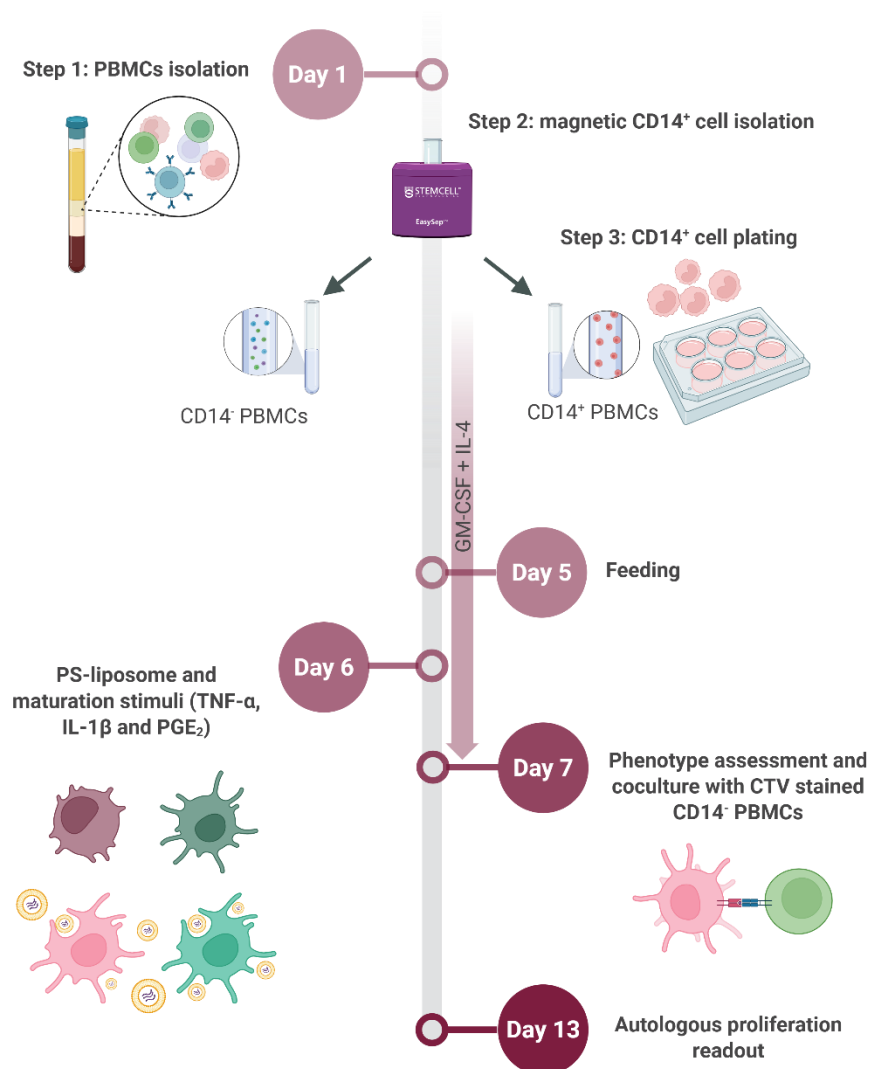


Figure 6. Schematic workflow of the different experiments with monocyte derived DCs and CD14⁺ PBMCs fraction from MG and RA patients. PBMCs were collected by density gradient centrifugation from peripheral blood samples. CD14 positive monocytes were magnetically sorted and cultured media supplemented with GM-CSF and IL-4 to generate DCs. On day 5, fresh cytokine-supplemented medium was added, and on day 6, PS-liposomes were introduced to the designated conditions. Two hours later, a maturation cocktail consisting of TNF- α , IL-1 β , and PGE₂ was added to induce DC maturation. After 24h (day 7), DCs were harvested either for phenotypic analysis by flow cytometry or for functional assays. For the latter, DCs were cocultured with autologous CD14⁺ PBMC fraction, previously labelled with CTV. After a 6-day incubation period, CD4⁺ and CD8⁺ T cell proliferation was assessed by flow cytometry based on dye dilution. Figure created using BioRender.

8.4. Differentiation of human DCs derived from peripheral blood monocytes

Monocytes were thawed in pre-warmed R-10 media, composed of RPMI-164 media (Biowest) with 10% FBS (ThermoFisher Scientific), and washed twice in PBS (Oxoid Limited) at 400 x g for 5 minutes at RT. Viability and cell number were determined with 7-AAD (BD Biosciences) viability staining, together with the Accucheck Counting Beads (ThermoFisher Scientific) using flow cytometry (FACS Canto II, BD Biosciences). After that, cells were cultured in 24-well plates (SPL Life Sciences, Pocheon-si, South Korea) at a 10^6 cells/mL in complete X-VIVO 15 media (Lonza, Basel, Switzerland), supplemented with 2% male AB human serum (Dominique dutscher, Bernolsheim, France), 100 IU/mL penicillin (Normon SA), 100 µg/mL streptomycin (Laboratorio Reig Jofré), and 1000 IU/mL IL-4 and 1000 IU/mL GM-CSF (Prospecbio, Ness-Ziona, Israel) to obtain monocyte-derived DCs. The culture was maintained in an incubator set at 37 °C with a 5% CO₂ atmosphere for 6 days. On day 4, new media were replenished with cytokines stimulus.

8.5. DCs phenotype after PS-liposome capture

Human DCs were differentiated from monocytes during 6 culture days. On day 5, 1mM, 0.1mM, 0.02mM or 0.004mM of PS-loaded-liposomes or PS-empty-liposomes were added to assess the tolerogenic effect after 24 hours incubation. For control purposes, DCs were cultured in basal conditions, resulting in immature DCs (iDCs), or adding a cytokine cocktail which consisted of 1000IU/mL tumor necrosis factor α (TNF- α ; Immunotools), 2000 IU/mL interleukin-1 β (IL-1 β , Immunotools) and 1µM prostaglandin E2 (PGE₂; Cayman Chemical, Ann Arbor, MI, USA) for 24 hours to obtain mature DCs (mDCs). In addition, conditions with PS-liposomes were also stimulated with the maturation cocktail for 24 hours to evaluate their response in the presence of a pro-inflammatory stimulus. The corresponding autoantigenic peptide was added to all DC conditions. After the 24 hour period of incubation, to harvest the attached DCs to the culture well, 500µL of Accutase (ThermoFisher Scientific) was added into each well for 20 minutes at 37°C. Afterwards, cells were washed at 400 x g for 5 minutes at RT. Cell viability was assessed with the viability staining, as aforementioned, by flow cytometry (FACS Canto II, BD Biosciences) (see **section 5**). Cells were stained for 20 minutes at 4°C with the following monoclonal antibodies listed in **Table 6**, to study the DC phenotype. After that, cells were washed in 1mL of PBS (Oxoid Limited) at 400 x g for 5 minutes at RT. For control purposes,

correspondent fluorescence-minus-one (FMO) staining was employed. Subsequent data analysis was carried out using FlowJo software (Tree Star).

Table 6. List of monoclonal antibodies used to determine human DCs phenotype

Target	Fluorophore	Species/Isotype	Clone	Use dilution *	Company
CCR7	PECy7	Rat IgG2a, κ	3D12	1:50	BD Biosciences
CD11c	APC	Mouse IgG1	BU15	1:250	Immunotools
CD14	PE	Mouse IgG1	18D11	1:500	Immunotools
CD184/ CXCR4	APCCy7	Mouse IgG2a, κ	12G5	1:250	BioLegend
CD25	PE	Mouse IgG1	HI25a	1:100	Immunotools
CD274/ PD-L1	PECy7	Mouse IgG2b, κ	29E.2A3	1:250	BioLegend
CD36	APCCy7	Mouse IgG2a, κ	5-271	1:250	BioLegend
CD40	APC	Mouse IgG1	HI40a	1:200	Immunotools
CD51/ Integrin $\alpha V\beta 5$	PE	Mouse IgG1, κ	P1F6	1:100	BioLegend
CD54	PECy7	Mouse IgG1, κ	HA58	1:300	BioLegend
CD86	FITC	Mouse IgG1	BU63	1:100	Immunotools
DC-SIGN	APC	Mouse IgG2a, κ	9E9A8	1:50	BioLegend
HLA-ABC	FITC	Mouse IgG2a	W6/32	1:100	Immunotools
HLA-DR	FITC	Mouse IgG2a	LT-DR	1:100	Immunotools
TIM-4	APC	Mouse IgG1, κ	9F4	1:250	BioLegend

* Prior to use, each antibody underwent a process of titration.

8.6. Proliferation response of autologous T lymphocytes upon stimulation by DCs

To assess the ability of human DCs to stimulate autologous T cell proliferation after engulfing PS-liposomes, detached DCs were co-cultivated with autologous CD14⁺ PBMCs. Before detaching the DCs on the fifth day of the differentiation culture, they were preloaded with the corresponding autoantigen at a concentration of 20µg/mL, which was the same peptide loaded into the PS-liposomes.

At the same time, on day 5, autologous PBMCs were thawed in pre-warmed R-10 media, composed of RPMI-164 media (Biowest) with 10% FBS (ThermoFisher Scientific), and then washed twice with PBS (Oxoid Limited) at 400 x g for 5 minutes at RT. Viability and cell number were determined with viability staining and Perfect Count Microspheres (ThermoFisher Scientific) by flow cytometry (FACS Canto II, BD Biosciences), as mentioned previously. For *in vivo* PBMCs labelling, we used the CellTrace™ Violet cell proliferation kit (CTV, ThermoFisher Scientific), which allows multiple generations to be traced by flow cytometry showing low cytotoxicity and no effect on the proliferative capacity or biology of the cells. The CTV stain readily crosses the plasma membrane and covalently binds to all free amines inside the cell. As the cells divide, the CTV stain is diluted evenly, allowing the different generations to be identified by flow cytometry. To achieve optimal staining of 8×10^6 PBMCs, we diluted them in PBS (Oxoid Limited) at 1×10^6 cells/mL and mixed them with 0.5µL mM CTV stock solution to obtain a working concentration of 0.31µM CTV. The mixture was incubated at 37°C for 20 minutes. Afterwards, unbound dye was quenched with R-10 medium and then cells were centrifuged at 400 x g for 5 minutes at RT. PBMCs were resuspended in a minimum of 5mL of R-10 and incubated 10 minutes at 37°C to allow ester hydrolysis. After that, cells were washed with PBS (Oxoid Limited) at 400 x g for 5 minutes at RT and then resuspended in X-VIVO 15 complete media. Viability and cell number were assessed by flow cytometry (FACS Canto II, BD Biosciences) (see **section 5**). Stained PBMCs were finally harvested at a final concentration of 10^6 cells/mL and 100,000 cells/well were seeded in a 96-well round bottom plates (ThermoFisher Scientific).

On the sixth day of the DC differentiation protocol, DCs were harvested from the 24-well plates (SPL Life Sciences) using Accutase (ThermoFisher Scientific), as aforementioned (see **section 8.4**). Viability and cell number were tested with the viability staining and perfect Count Microspheres using flow cytometry (FACS Canto II, BD Biosciences) (see **section 5**). DCs were harvested and diluted with X-VIVO 15

complete media to a final concentration of 0.1×10^6 cells/mL. To finally achieve a DCs:PBMcs ratio of 1:10, we cocultured 10,000 DCs per well in the 96-well round bottom plate (ThermoFisher Scientific) where PBMcs were seeded the previous day. For each sample, triplicate cultures were established under the following conditions: basal conditions as a negative control, or with the addition of 50ng/mL Phorbol 12-Myristate 13-Acetate (PMCA, Sigma) and 500ng/mL Ionomycin (Sigma) as a positive control.

Following a cultivation period of six days, maintained at 37°C and 5% CO₂, T cell proliferation was assessed differentiating both, CD4 and CD8 T cell compartment. Each well, that contained cells, in the 99-well plate was washed with PBS (Oxoid Limited) at 400 x g for 5 minutes at RT. Then cells were stained, during 20 minutes at 4°C, with the viability staining 7-AAD (BD Biosciences), and the monoclonal antibodies listed in **Table 7**. Finally, cells were again washed with PBS (Oxoid Limited) as abovementioned and suspended in 150µl PBS (Oxoid Limited) per well. Samples were acquired with FACS LSR Fortessa (BD Biosciences), and subsequent data analysis was carried out using FlowJo software (Tree Star).

Table 7. List of monoclonal antibodies used to assess T cell proliferation

Target	Fluorophore	Species/Isotype	Clone	Use dilution *	Company
CD3	PE	Mouse monoclonal IgG1, κ	UCHT-1	1:100	Immunotools
CD4	APC	Mouse IgG2a	IT4	1:100	Immunotools
CD8	FITC	Mouse IgG1, κ	MEM-31	1:100	Immunotools

* Prior to use, each antibody underwent a process of titration.

8.7. Culture of SF derived DCs and phenotype assessment

When it was feasible, SFMCs were thawed in pre-warmed R-10 medium, consisting of RPMI-164 media (Biowest) with 10% FBS (ThermoFisher Scientific), and washed twice in PBS (Oxoid Limited) at 400 x g for 5 minutes at RT. Cell viability and count

were determined using 7-AAD (BD Biosciences) viability staining and Accucheck Counting Beads (ThermoFisher Scientific), as outlined in **section 5**. When possible, immune cell subsets of SFMCs were assessed by flow cytometry using the monoclonal antibodies listed in **Table 8**. List of monoclonal antibodies used to detect the immune cell subsets in human PBMCs and SFMCs. Additionally, the percentage of CD14-positive cells was evaluated using an anti-human CD14 PE antibody (Immunotools) with a 15-minute incubation at RT. The CD14 positive fraction was isolated as described in **section 8.2**. and subsequently plated under the same conditions as monocytes from PBMCs, detailed in **section 8.4**. The CD14 negative fraction of SFMCs was cryopreserved in FBS (ThermoFisher Scientific) containing 10% DMSO (Sigma) at a concentration of $15\text{-}20 \times 10^6$ cells/mL.

Table 8. List of monoclonal antibodies used to detect the immune cell subsets in human PBMCs and SFMCs

Target	Fluorophore	Species/Isotype	Clone	Use dilution *	Company
CD11c	PECY7	Mouse BALB/c IgG1, κ	B-ly6	1:250	BD Biosciences
CD14	BV711	Mouse IgG1, κ	63D3	1:50	BioLegend
CD19	BV785	Mouse IgG1, κ	H1B19	1:250	BioLegend
CD3	BV650	Mouse IgG1, κ	UCHT1	1:100	BioLegend
CD4	BV570	Mouse IgG1, κ	RPA-T4	1:40	BioLegend
CD68	BV421	Mouse IgG2b, κ	Y1/82A	1:100	BioLegend
CD8	APCCY7	Mouse IgG1, κ	SK1	1:500	BioLegend

* Prior to use, each antibody underwent a process of titration.

After 5 days of culture, PS-MCV-liposomes or PS-empty-liposomes were added to evaluate the tolerogenic effect following 24 hours of incubation. For control conditions, synovial fluid monocyte-derived DCs were cultured under basal conditions, resulting in putative iDCs, or adding a cytokine cocktail which consisted

of 1000 IU/mL TNF- α (Immunotools), 2000 IU/mL IL-1 β (Immunotools) and 1 μ M PGE₂ (Cayman Chemical, Ann Arbor, MI, USA) for 24 hours to obtain putative mDCs. Furthermore, PS-liposome-treated conditions were also stimulated with the maturation cocktail for 24 hours to assess the response in the presence of a pro-inflammatory stimulus. Cells were detached using Accutase (ThermoFisher Scientific), as described in **section 8.4**. Viability was measured using viability staining, followed by flow cytometry analysis (FACS Canto II, BD Biosciences), as detailed in **section 5**. To study the DC phenotype, cells were stained for 20 minutes at 4°C with the monoclonal antibodies listed in **Table 6** (see **section 8.5**).

8.8. Proliferation SF derived DCs and SFMCs

On the fifth day of SFDCs differentiation, cells were preloaded with the MCV peptide at a concentration of 20 μ g/mL. At the same time, the autologous CD14 negative fraction of the SFMCs was thawed in pre-warmed R-10 media, consisting of RPMI-164 media (Biowest) with 10% FBS (ThermoFisher Scientific). The cells were then washed twice in PBS (Oxoid Limited) at 400 x g for 5 minutes at RT. Cell viability and count were determined using 7-AAD viability staining and Accucell Counting Beads (ThermoFisher Scientific), as described in **section 5**. For *in vivo* SFMC labelling, the CTV Cell Proliferation Kit (ThermoFisher Scientific) was used in the same manner as with PBMC staining (see **section 8.6**). Viability and cell number were again assessed by flow cytometry (FACS Canto II, BD Biosciences) (see **section 5**). Labelled SFMCs were then resuspended at a final concentration of 1 x 10⁶ cells/mL, and 100,000 cells/well were plated in 96-well round-bottom plates (ThermoFisher Scientific).

On the sixth day of the SFDCs differentiation protocol, cells were harvested from the 24-well plates (SPL Life Sciences) using Accutase (ThermoFisher Scientific), as previously described (see **section 8.4**). Viability and cell number were assessed as described in **section 5**. The harvested SFDCs from each condition were diluted with X-VIVO 15 complete media to a final concentration of 0.1 x 10⁶ cells/mL and plated 10,000 SFDCs per well in the 96-well round bottom plate (SPL Life Sciences). For each sample, triplicate cultures were established under the following conditions: basal conditions as a negative control, or with the addition of 50 ng/mL Phorbol 12-Myristate 13-Acetate (PMCA, Sigma) and 500 ng/mL Ionomycin (Sigma) as a positive control.

After a six-day cultivation period at 37°C and 5% CO₂, T cell proliferation was evaluated, distinguishing between the CD4 and CD8 T cell compartments using the

monoclonal antibodies listed in **Table 7** (see **section 8.6**). Samples were acquired with FACS LSR Fortessa (BD Biosciences), and subsequent data analysis was performed with FlowJo software (Tree Star).

9. HLA class II peptide presentation after DCs cocultured with PS-liposomes

To further elucidate the underlying mechanism of action, a key question was whether the autoantigen encapsulated within PS-liposomes was being processed and presented by DCs to T cells via MHC molecules. To address this, immunoprecipitation techniques were employed to isolate the peptide:MHC complexes from the surface of DCs (**Figure 7**). This approach was followed by tandem mass spectrometry (MS/MS) analysis to identify the specific peptides being presented, allowing for a deeper understanding of the antigen presentation pathway and its potential role in immune modulation.

9.1. Monocyte isolation from buffy coats and lysate obtaining

Due to the high number of DCs required for the following protocol, the starting material for each donor was the buffy coat which contain the same number of leukocytes as the original blood donation, but in higher concentrations. The blood was poured into 50mL falcon, and the buffy coat bag was then washed with 50mL of PBS (Fresenius Kabi, Huis ter Heide, Netherlands) supplemented with 0.4% of human serum albumin (HSA; Prothya Biosolutions, Amsterdam, Netherlands) in order to dilute the blood at a 1:1 ratio. PBMCs were acquired using Ficoll Paque (GE Healthcare Life Sciences) density gradient centrifugation. The process involved centrifuging at 400 x g without brake for 25 minutes at RT, after adding two volumes of diluted blood to one volume of Ficoll solution carefully ensuring meticulous separation of the two phases. Following centrifugation, the PBMCs layer was aspirated using a Pasteur pipette. Cells were resuspended in PBS (Fresenius Kabi) supplemented with 0.4% HSA (Prothya Biosolutions) medium. Viability and cell number assessment was conducted using a disposable cell counting slide (Glasstic slide, Kova International, Amsterdam, Netherlands) and Trypan blue.

The isolated PBMCs were resuspended in PBS supplemented with 0.4% HSA, 1% P/S and 2mM EDTA and the CD14 positive fraction was isolated following the CD14 MicroBeads (Miltenyi) manufacturer's instructions and using the LS columns (Miltenyi). Cell number was again evaluated using the disposable cell counting slide

(Kova International) and Trypan blue. Monocytes were suspended to a concentration of 10^6 cells/mL using the CellGro GMP DC Medium (Sartorius CellGenix GmbH, Freiburg, Germany) supplemented with 1% penicillin (Normon SA) and streptomycin 800 IU/mL IL-4 and 1000 IU/mL GM-CSF (Prospecbio) to obtain DCs. The culture was maintained in an incubator set at 37 °C with a 5% CO₂ atmosphere for 5 days. After this time, iDCs were detached from the plate with trypsin (ThermoFisher Scientific) trying not to exceed the 15 minutes incubation at 37 °C. iDCs number was evaluated and cells were replated, this time at a 2.5×10^6 cells/mL concentration in CellGro GMP DC Medium (Sartorius CellGenix GmbH) supplemented with 1% penicillin (Normon SA) and streptomycin 800 IU/mL IL-4 and 1000 IU/mL GM-CSF (Prospecbio). The appropriate stimuli were added, either AChR peptide or SPACER peptide at a final concentration of 20 µg/mL; or PS-AChR liposomes or PS-SPACER liposomes at a final concentration of 1mM. After 5h of incubation period, the maturation stimuli was added, which consisted of HSA (Prothya Biosolutions) supplemented with 2% of Lipopolysaccharide (LPS, Milipore, Burlington, MA, USA).

After 24 hours, mDCs were harvested using cold CellGro GMP DC Medium (Sartorius CellGenix GmbH). 100,000 cells from each condition were stained for 20 minutes at 4°C to study the DC phenotype with the following monoclonal antibodies: CCR7 PEcy7, CD11c APC, CD14 PE, CXCR4 APCCy7, CD25 PE, PD-L1 PEcy7, CD36 APCCy7, CD40 APC, CD54 PEcy7, CD86 FITC, HLA-DR FITC and TIM4 APC, listed previously in **Table 6**. The rest of the cells were immediately lysed during 30 minutes at 4°C with 500µl of lysis buffer composed of 10mM Tris pH 8.0, 0.25% octyl-b-D glucopyranoside, 1% sodium deoxycholate and EDTA 10mM. To end, the lysates were centrifuged at 20,000 x g for 15 minutes at 4°C.

9.2. Anti HLA-II antibodies coupling to CNBr-sepharose

The required amount of CNBr-activated Sepharose 4B (Cytiva, Marlborough, Massachusetts, USA) was weighted to achieve a coupling density of 2mg/ml in 10mL of 1mM HCl and incubated for 20 minutes at RT. Following incubation, the Sepharose was centrifuged at 850 x g for 5 minutes and washed again with 1mM HCl. The coupling buffer, consisting of 0.1 M NaHCO₃ and 0.5 M sodium chloride (NaCl) pH 8.3, was then added to the Sepharose, followed by another centrifugation at 850 x g for 5 minutes. Subsequently, 20mg of protein on interest—specifically, L243 and SPV-L3 antibodies— was added along with the coupling buffer, and the mixture was incubated overnight at 4°C under continuous rotation. The following day, the antibody-coupled Sepharose was washed at 850 x g for 5 minutes. The supernatants

were collected and analysed using a NanoDrop spectrophotometer to quantify the unbound protein. Subsequently, a stopping buffer containing 0.2 M glycine at pH 7.5 was added, and the mixture was incubated for 1 hour at RT. This step was repeated twice, and afterwards a washing process was subsequently repeated three times. Each cycle involved washing the Sepharose first with the coupling buffer, followed by acetate buffer— 0.1M sodium acetate, 0.5M NaCl and 0.048M citric acid at pH 4.0—, and finally with 10mM TRIS buffer. All washes were conducted by centrifugation at 850 x g for 5 minutes. Finally, L243- and SPVL3-coupled Sepharose was resuspended in 10 mM TRIS buffer at pH 7.5 and stored at 4°C until further use.

9.3. HLA purification and peptide elution

The proper amount of the previously antibody-coupled CNBr-sepharose beads, either L243 or SPV-L3, was taken to ensure a proper binding to the peptide:MHC class II complexes. After washing the beads with TRIS 1mM and EDTA 10mM buffer, these were mixed with the cell lysates and incubated overnight at 4°C ‘end-over-end’, using a laboratory rotator. L243 and SPV-L3 Sepharose beads were then washed 2 times with lysis buffer and 5 times with 10 mM Tris-HCl (pH 8.0) to remove non-specific bound material (**Figure 7**). Peptide:MHC complexes were eluted using 500 mL 10% acetic acid for 10 minutes at RT. Then the samples were centrifuged at 400 x g for 5 minutes at RT and the supernatant was transferred to low-binding 1.5 mL Eppendorf tubes and heated for 15 mins at 70°C to dissociate the peptide:MHC complexes. Samples were desalted and concentrated using 1.9 µm C18 STAGE Tips (Dr. Maisch GmbH, Amersfoort, the Netherlands). The STAGE tips were previously equilibrated with 100% acetonitrile and then washed with 1% formic acid to promote peptide binding. Samples were loaded onto the STAGE tips and washed once with 1% formic acid and once with 1% formic acid supplemented with 5% acetonitrile to remove contaminants.

9.4. Mass spectrometry

Before running the samples, peptides were eluted from the STAGE tips using 60µL of 1% formic acid with 30% acetonitrile and concentrated to a final volume of 5µL by vacuum centrifugation at 45°C with SpeedVac centrifuge (Eppendorf Concentrator Plus, Eppendorf, Hamburg, Germany). The eluate was introduced directly into an Orbitrap Fusion Tribrid mass spectrometer (ThermoFisher Scientific Inc., Bremen, Germany) via a nanoelectrospray source set at a spray voltage of 2.15 kV. Higher-energy collisional dissociation was conducted in top-speed mode with 3-second

cycles (400–1500 m/z, resolving power of 120,000). To ensure high mass accuracy, the mass spectrometer was regularly calibrated according to the manufacturer's recommendations. Peptides were identified using Proteome Discoverer 1.4 (Thermo Scientific, Bremen, Germany) and raw Xcalibur data files were screened against the UniprotKB non-redundant protein 25.H_sapiens.fase database. Peptide identifications were separated in high/medium or low confidence depending on the False Discovery Rate (FDR), automatically calculated by the workflow, lower or higher than the 5% established threshold, respectively. For HLA-DR immunopeptidome analysis, the list of proteins with the Gene Ontology (GO) term and peptides detected in each condition was used.

To visualize and compare the relative abundance of proteins identified in unstimulated matured DCs conditions, two heatmaps were generated using the R programming language (version 4.2.2). The input data consisted of a merged table containing all identified proteins and their corresponding spectral counts across both conditions. After data importation using the readr package, non-numeric columns were excluded to construct a numerical matrix of protein counts. Protein identifiers were assigned as row names. Missing values, corresponding to proteins not detected in a given condition, were replaced with zeros. The combined matrix was scaled by row (protein-wise normalization or z-score normalization) and the heatmap was constructed using the pheatmap package. A custom colour gradient ranging from white to light blue to dark blue was defined representing low, intermediate, and high abundance levels, respectively. In addition, to reduce complexity and focus on the most abundant proteins, the matrix was sorted in descending order based on the average counts per protein, and the top 50 proteins were selected for visualization. The heatmap was constructed using the pheatmap package. A custom colour gradient ranging from red to white to blue was defined representing low, intermediate, and high abundance levels, respectively. Row and column clustering was always performed using Euclidean distance and complete linkage. To improve readability, displayed values within the heatmap corresponded to raw count values, allowing for intuitive interpretation of protein distribution patterns between conditions. Using GO terms of the HLA-DR ligandome, a protein class supergrouping was performed using the open-access web server PANTHER 19.0 (<https://pantherdb.org>, accessed on 12 September 2024) [398]. For specific peptide identification in those matured DCs conditions treated with peptides or PS-loaded-liposomes, the GO term for AChR peptide was: Acetylcholine receptor subunit alpha OS=Homo sapiens OX=9606 GN=CHRNA1 PE=1 SV=3; and the term for SPACER

peptide was: A disintegrin and metalloproteinase with thrombospondin motifs 13
OS=Homo sapiens OX=9606 GN=ADAMTS13 PE=1 SV=1.

9.5. DNA extraction for HLA genotyping

To determine the HLA typing of each control subject participating in the peptide HLA class II presentation study, the CD14-negative fraction of PBMCs obtained after magnetic separation was collected. The cells were processed following the manufacturer's instructions using the DNeasy Blood and Tissue Kit for DNA isolation (Qiagen, Hilden, Germany). After DNA extraction, the samples were stored at -20°C until they were sent to GenDX (Utrecht, The Netherlands) for HLA genotyping analysis.

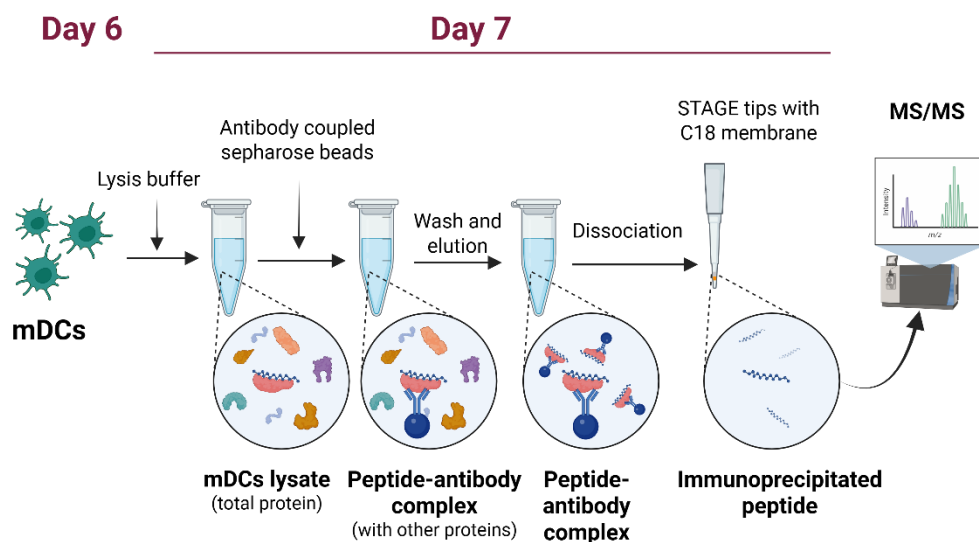


Figure 7. Schematic protocol of HLA class II:peptide complex isolation from mDCs for MS/MS analysis. MDCs, unstimulated or stimulated with peptides or PS-loaded-liposomes, were lysed and incubated overnight with anti-HLA-DR or anti-HLA-DQ antibodies covalently bound to Sepharose beads. Following extensive washing to remove unbound material, HLA:peptide complexes were eluted and subjected to acid treatment and heat to dissociate the peptides. The resulting peptide fractions were loaded onto C18 membrane-containing STAGE tips, where they were retained, washed, and stored until elution and injection for tandem mass spectrometry (MS/MS) analysis.

10. Whole PBMCs and SFMCs

PS-liposomes are designed to mimic apoptotic bodies, a crucial component of the physiological process of maintaining homeostasis. DCs and M ϕ are well-documented for their capacity to recognize and clear apoptotic bodies through specific receptors. However, to expand our understanding of whether other immune cell subsets also possess the ability to interact with PS-liposomes and through which receptors, PBMCs obtained from either patients or healthy controls or SFMCs from RA patients, were cultured with fluorescently labelled PS-liposomes. Surface markers were then analysed to further investigate the interactions and receptor-mediated mechanisms involved in this process.

10.1. Peripheral Blood Mononuclear Cell isolation

Blood draw from healthy donors and the subsequent PBMCs isolation was performed as indicated in **section 8.1**. SFMC were isolated as indicated in **section 8.3**. Once the PBMCs or SFMCs were collected, cells were stained with 7-AAD (BD Biosciences), and together with the Accucheck Counting Beads (ThermoFisher Scientific) the viability was assessed using flow cytometry (FACS Canto II, BD Biosciences) (see **section 5**). PBMCs were cryopreserved in FBS (ThermoFisher Scientific) with 10% DMSO (Sigma) at a concentration of $15\text{--}20 \times 10^6$ cells/mL.

10.2. Evaluation of the PS-liposome effect in PBMCs and SFMCs

The PBMCs from healthy donors, PBMCs CD14⁺ from MG and AR patients or SFMCs were thawed in pre-warmed R-10 media, composed of RPMI-164 media (Biowest) with 10% FBS (ThermoFisher Scientific), and washed twice in PBS (Oxoid Limited) at $400 \times g$ during 5 minutes at RT. To assess the different immune cell subsets present in the sample, the phenotype was studied using the anti-human antibodies listed in **Table 8**. Viability and cell number assessment was conducted using Annexin V PE staining (Immunotools) and 7-AAD (BD Biosciences), followed by incubation at RT for 15 minutes, and Accucheck Counting Beads (ThermoFisher Scientific) using flow cytometry (FACS Canto II, BD Biosciences) (see **section 5**). After that, PBMCs were cultured in 24-well plates (Labclinics) at 10^6 cells/mL in complete X-VIVO 15 media (Lonza), supplemented with 2% male AB human serum (Dominique dutscher), 100 IU/mL penicillin (Normon SA), 100 $\mu\text{g/mL}$ streptomycin (Laboratorio Reig Jofré). Subsequently, fluorescently-labelled-PS-liposomes or non-fluorescent PS-liposomes were added. For control purposes, PBMCs were cultured in basal conditions without

PS-liposomes. Cells were maintained in an incubator set at 37 °C with a 5% CO₂ atmosphere for 24h.

For PBMCs harvesting, the supernatant was removed from each well and, 500µL of Accutase (ThermoFisher Scientific) was added into each well for 20 minutes at 37°C to facilitate the detachment of potentially adherent cells. Afterwards, cells were washed at 400 x g for 5 minutes at 4°C with 1mL of PBS (Oxoid Limited) supplemented with 5% of human serum albumin (Grifols, Barcelona, Spain), previously vortexed for 15 seconds to remove any weak attachment of the PS-liposomes to the cell membrane. This washing step was repeated four additional times, always maintaining the cells at 4°C. Finally, cells were stained with the anti-human antibodies listed in **Table 8**. After the incubation at 4°C for 20 minutes, cells were analysed using flow cytometry (FACS LSR Fortessa, BD Biosciences). To assess regulatory cytokines production, the IL-10 secreting assay detection kit (Miltenyi) and the LAP (TGF-β1) anti-human antibody (Miltenyi) were added to the immune cell subsets staining mix according to the manufacturer's instructions.

To study PS receptors expressed in the immune cell subsets that can interact with PS-liposome, the following monoclonal antibodies were added to the cytometry staining mix (**Table 9**).

Table 9. List of monoclonal antibodies used to determine PS-receptors in different immune cell subsets

Target	Fluorophore	Species/Isotype	Clone	Use dilution *	Company
TIM-1	PECy7	Mouse IgG1, κ	1D12	1:100	BioLegend
TIM-4	APC	Mouse IgG1, κ	9F4	1:250	BioLegend
CD19	BV785	Mouse IgG1, κ	H1B19	1:250	BioLegend
CD14	BV711	Mouse IgG1, κ	63D3	1:50	BioLegend
CD3	BV650	Mouse IgG1, κ	UCHT1	1:50	BioLegend
CD4	BV570	Mouse IgG1, κ	RPA-T4	1:40	BioLegend
Axl	BV510	Mouse IgG1, κ	175128 (RUO)	1:100	BD Biosciences
TIM-3	BV421	Mouse IgG1, κ	F38-2E2	1:100	BioLegend
Integrin αβv5	PE	Mouse IgG1, κ	P1F6	1:250	BioLegend
CD36	APCCy7	Mouse IgG2a, κ	5-271	1:250	BioLegend

* Prior to use, each antibody underwent a process of titration.

11. Hemocompatibility tests

In order to ensure safety in the future clinical trial some regulatory parameters have to be evaluated. Here we examined the quality of the red blood cells (RBC) after the incubation with PS-liposomes assessing three different parameters: erythrocyte aggregation, haemoglobin oxidation and osmotic fragility.

Blood samples from six healthy controls (3 females and 3 males) were obtained using BD Vacutainer Sodium Heparin tubes (BD Biosciences) until reaching a total volume of 10 mL. Without diluting, 500 μ l of fresh blood were taken to incubate with 50 μ l of PBS, for control purposes, or with the same amount of PS-AChR-liposomes. In this case, MG PS-AChR-liposomes were used to assess the effect in three different concentrations (**Table 10**). After 5, 30 and 120 minutes of incubation, 2 μ l of this mixture were diluted using Hanks' Balanced Salt Solution (HBSS) until reaching a final volume of 1000 μ l, and subsequently the sample was properly mixed and acquired by the Invitrogen™ Attune™ NxT Acoustic Focusing Cytometer (ThermoFisher Scientific). Erythrocyte morphology was checked looking for abnormally shaped RBC by Attune CytPix Flow Cytometer (ThermoFisher Scientific).

The erythrocyte aggregation was calculated assessing the acquired number of singlets taking the advantage of the different light absorption of erythrocytes, by collecting the blue and violet SSC simultaneously. The haemoglobin oxidation was tested by analysing the violet SSC absorption at 413nm evaluating the potential variations over time.

To assess the osmotic fragility, the erythrocyte light-scatter properties were tested under osmotic pressure according to the method of Beutler [399]. To do that, 500 μ l of the blood incubated with the different conditions of PS-AChR-liposomes was introduced to a hypotonic solution (NaCl 0.55%). In this media water penetrated inside the erythrocyte which swells, until the capacity of the cell membrane is exceeded and finally bursts. The total number of erythrocytes was monitored over time in every condition to detect variances among conditions.

Table 10. List of concentrations of PS-AChR-liposomes used for regulatory experiments

Concentration of PS-AChR-liposome batch (mM)	Concentration use (mM)	Purpose
1.96	0.18	To mimic concentration reached in the bloodstream of a human after the IV administration
15.83	1.4	Seven times more concentrated
31.1	2.82	Fifteen times more concentrated

12. Detection of cytokines of the cytokine storm release

Given that PS-liposomes are intended for IV administration, a critical aspect of their safety profile is to assess their potential impact on systemic inflammation. To evaluate the effects of PS-liposomes in the bloodstream, a key regulatory measure was undertaken to determine whether their presence induces an overproduction of inflammatory cytokines, which could potentially cause systemic damage to host cells.

First, blood samples from six healthy controls (3 females and 3 males) were obtained using EDTA Vacutainer tubes (BD Biosciences) until reaching a total volume of 10 mL. Without diluting, 500µl of fresh blood were taken to incubate with 50 µl of PBS or LPS, for control purposes, or with the same amount of PS-AChR-liposomes (**Table 10**). At the beginning of the experiment (0h timepoint) and after 4-, 24- or 48 hours of incubation, plasma was collected after centrifuging the blood at 2000 x g for 10 minutes at 4°C. Plasma samples were stored at -20° for further use. To assess the secretion of the G-CSF, GM-CSF, IFN alpha, IFN gamma, IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-18, TNF alpha, TNF beta cytokines and the IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 alpha (CCL3), MIP-1 beta (CCL4) chemokines, the ProcartaPlex™ Human Cytokine Storm Panel 21plex (ThermoFisher Scientific) was used according to the manufacturer's instructions. This immunoassay is based on the principles of a sandwich ELISA, using matched pairs of highly specific antibodies binding to different epitopes of one protein to quantitate all protein targets simultaneously. In the multiplexed assay, each spectrally unique bead is labelled with antibodies specific for a single target protein, and bound proteins are identified with

biotinylated antibodies and streptavidin–R-phycoerythrin (RPE). The conjugation of protein-specific antibodies to a distinct bead allows for analysis of multiple targets in a single well. Each well was read using the Luminex 200™ System (ThermoFisher Scientific) and the data was analysed with the ProcartaPlex Analysis App (ThermoFisher Scientific).

13. Dose-response evaluation

A potency assay is a crucial component of the regulatory experiments required to obtain approval to initiate clinical trials. This assay must be designed to demonstrate a dose-response effect, based on the mechanism of action of the new therapy, which is essential to empirically determine the potency of each newly produced batch of PS-liposomes. By establishing a reliable dose-response relationship, the potency assay ensures consistency and efficacy across production batches, thus meeting regulatory standards for access to clinical trials.

13.1. TANOUE B cell line culture

The TANOUE B cell line (Leibniz Institute DSMZ, Braunschweig, Germany) derived from the peripheral blood of an 11-year-old boy with acute lymphoblastic B cell leukaemia, was selected for its favourable characteristics, particularly its ability to grow in suspension, making it suitable for cell culture experiments. To maintain the cell line, TANOUE cells were seed at $1\text{--}2 \times 10^6$ cells/mL in a 75cm² flask (SPL Life Sciences) and split twice a week in a 1:3 ratio. The media used to culture them was composed of 90% RPMI-164 (Biowest), 10% heat inactivated FBS (ThermoFisher Scientific) and 100 IU/mL penicillin (Normon SA), 100 µg/mL streptomycin (Laboratorio Reig Jofré). To study PS-liposome interaction, NBD-labelled PS-loaded-liposomes were included together at 0, 0.1, 0.5 and 1mM concentration. After 24 hours of incubation, cell viability was assessed with the viability staining, as aforementioned (see **section 5**), and NBD positivity by FITC fluorescence emission by flow cytometry (FACS Canto II, BD Biosciences). Subsequent data analysis was carried out using FlowJo software (Tree Star).

13.2. Whole PBMCs culture

PBMCs from control subjects were isolated as described in **section 8.1** and stored cryopreserved in FBS (ThermoFisher Scientific) with 10% DMSO (Sigma) at a concentration of $15\text{--}20 \times 10^6$ cells/mL. After thawing the PBMCs, the whole fraction

was directly plated in complete X-VIVO 15 media (Lonza), supplemented with 2% male AB human serum (Dominique Dutscher), 100 IU/mL penicillin (Normon SA), 100 µg/mL streptomycin (Laboratorio Reig Jofré).

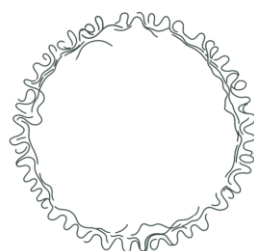
13.3. Tolerogenic marker TOLX expression assay

For reasons of confidentiality and industrial secrecy, from now on, the marking used for the potency assay will be called: TOLX. Cells of choice were plated in 24-well plates (SPL Life Sciences) at 10^6 cells/mL using their respective complete media. Subsequently, PS-AChR-liposomes were added in an increasing gradient of concentrations, composed by 0 mM, 0.001mM, 0.01mM, 0.1mM, 0.5mM, 1mM and 2mM. After 24h of incubation at 37 °C with a 5% CO₂ atmosphere, cells were harvested and washed at 400 x g for 5 minutes at 4°C with 1mL of PBS (Oxoid Limited) supplemented with 5% of human serum albumin (Grifols, Barcelona, Spain). After that, cells were stained with the following anti-human antibodies for 20 minutes at 4°C: CD19 BV785 and TOLX marker. Viability was conducted using 7-AAD. Finally, TOLX surface expression on B cells was analysed using flow cytometry (FACS LSR Fortessa, BD Biosciences).

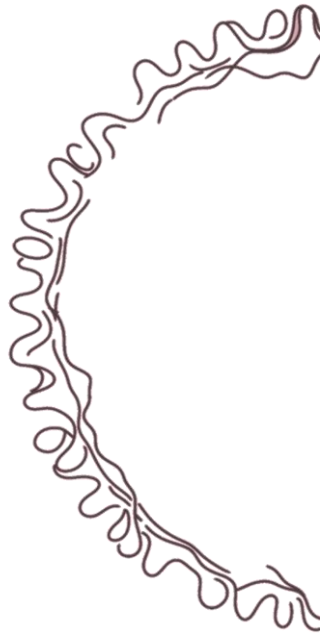
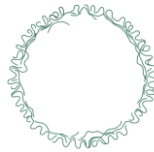
14. Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) or standard error of the mean (SEM). Statistical analyses were conducted using the Prism 9.0 software (GraphPad Software Inc., San Diego, CA) and R-studio v4.3.2 (RStudio, PBC, Boston, MA). Normality of the data was verified using the Shapiro-Wilk test, and homogeneity of variances between groups was evaluated using the F-test. Comparisons between two unpaired groups were performed using either a two-tailed unpaired Student's t-test (for normally distributed data) or the non-parametric Mann-Whitney U test (for non-normally distributed data). A parametric t-test or a non-parametric Wilcoxon signed rank test was used for paired data depending on the distribution. When comparing relativised conditions to the respective control condition, one sample Wilcoxon signed-rank test was performed. To evaluate the interaction of two independent variables on a dependent variable, two-way ANOVA with Tukey's multiple comparisons was applied. When data did not meet normality assumptions, the Kruskal-Wallis test followed by Dunn's multiple comparisons was used. For matched data involving more than two conditions, a mixed-effects model (REML) with Tukey's or Dunnett's post hoc test was employed. For all tests, a two tailored p-value of $\leq$

0.05 was considered statistically significant. Levels of significance are indicated as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$.



4



RESULTS

SECTION I. *In vivo* and *ex vivo* evaluation of biodistribution and capture biokinetics of PS-liposomes

The research on the biodistribution and fate of a new therapy is essential for predicting and assessing the drug delivery, given their implications in pharmacokinetics, efficacy and potential toxicity profiles. In addition, the biodistribution of nanocarriers and associated toxicity risks are expected to vary significantly depending on their routes of administration, dosage and intrinsic properties. Moreover, the efficacy of the NP's-based therapy also may vary depending on the administration, since its uptake by immune cells can be altered modifying the mechanism of action.

1. Fluorescently labelled PS-liposomes encapsulate human peptides

PS-liposomes were generated with a specific molar ratio of DOPS/DMPC/CH to emulate an apoptotic body presenting the PS on its surface. To produce the fluorescent PS-liposomes, DOPE-AF750 or NBD staining was introduced in the process of PS-liposome elaboration. After their production, every batch was characterised in terms of lipid concentration, diameter size, Pdl – measuring the distribution of sizes within the PS-liposome sample–, Z-potential –measuring the surface charge– and encapsulation efficiency of the correspondent peptide. The physicochemical characteristics of the PS-liposomes used in this section are shown in **Table 1**.

To guarantee efficient phagocytosis, the PS-liposomes were consistently manufactured with a minimum diameter of 500 nm, and their concentration was maintained at approximately 30 mM. Some batches of NBD-labelled PS liposomes lack lipid concentration measurement due to non-standard data compilation. The negative Z-potential was confirmed, ensuring their tolerogenic properties.

Table 11. Physicochemical features of PS-liposomes used in SECTION I

Liposome nomenclature	Number of batches	Lipid concentration (mM)	Diameter size (nm)	Pdl	Z-potential (mV)	Encapsulation efficiency (%)
DOPE-AF750-PS-AChR-liposomes	1	23.08	737.5	0.31	-21.1	97.14
DOPE-AF750-PS-liposomes	2	23.90	720.50 ± 6.40	0.28 ± 0.04	-26.30 ± 0.60	NA
NBD-PS-AChR-liposomes	1	29.83	578.30	0.33	-22.3	96.04
NBD-PS-empty-liposomes	3	~ 30	788.80 ± 282.40	0.27 ± 0.13	-26.50 ± 3.90	NA
NBD-PS-Ins-liposomes	3	~ 30	894.70 ± 256.70	0.27 ± 0.09	-28.80 ± 5.20	84.60 ± 18.9

Data presented as mean ± SD; DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; NA: not applicable; NBD; nitrobenzoxadiazole; Pdl: polydispersity index.

2. AF750-labelled PS-liposomes exhibit targeted organ distribution after different administration routes

In this study, to gain mechanistic insight, we first systematically compared four different administration routes – IP, ID, IV, and IN– using, in this first attempt, empty DOPE-AF750-labelled PS-liposomes in prediabetic NOD mice. Although the fluorescent signal is not comparable between routes due to variations in the maximum administered volume permitted, we could analyse and compare the resulting distinct biodistribution patterns 1-, 3-, 6- and 24-hours after the injection. This approach provided an initial overview of the biodistribution found in **Supplementary Figure 1** and facilitated a more detailed study of the fluorescence emission of DOPE-AF750-labelled PS-liposomes in each organ (**Figure 8**).

The IP administration route (**Figure 8A**) showed high fluorescence values 1h post-injection of DOPE-AF750-PS-liposomes, ranging from 185.85 to 112,540.86 RFU/grams of tissue. Notably, this signal was particularly prominent in organs proximal to the injection site, including the PAT, pancreas, or spleen. Remarkably, an accumulation in regional lymph nodes as MDLN and PLN was observed, with fluorescence peaks ranging from 376.30 to 40,945.77 RFU/g detected at the 3h timepoint, being higher than 1h timepoint. Furthermore, this trend persisted 6h after the injection, with fluorescence peaks between 310.96 and 61,555.86 RFU/g, indicating a slow biodistribution and accumulation in organs such as PAT, pancreas,

stomach, spleen, and regional lymph nodes. After 24h, the fluorescence signal decreased with peaks between 18.37 and 19,422.79 RFU/g. However, at this time point, it's noteworthy to mention that the maximum peaks were considerably higher than those obtained with other administration routes, indicating a slower biodistribution pattern in this route.

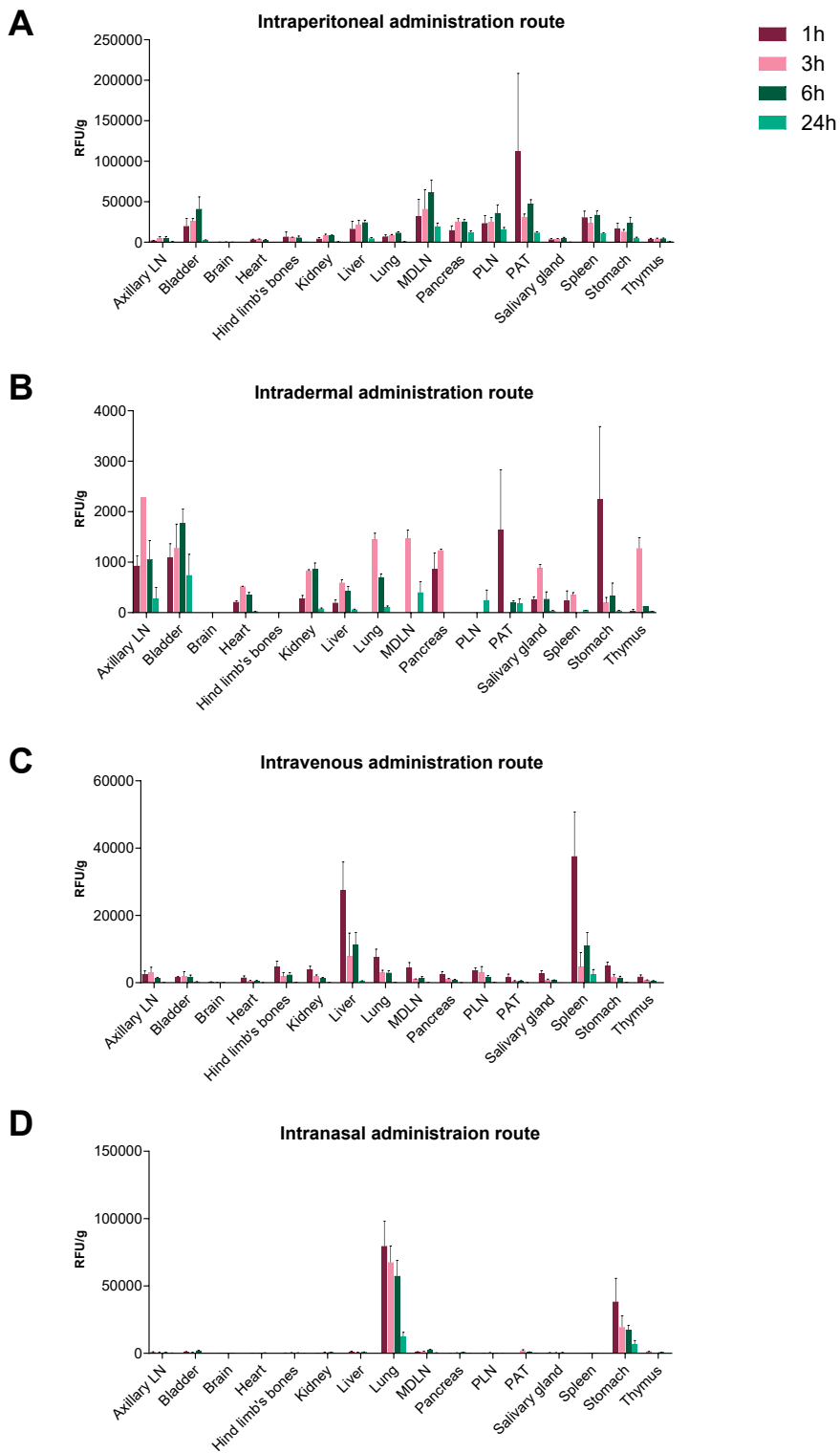


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Figure 8. PS-AF750-liposomes biodistribution after different administration routes in prediabetic NOD mice. Intensity of fluorescence in organs after IP administration (n=2-8) **(A)**, ID administration (n=2-3) **(B)**, IV administration (n=2-5) **(C)** and IN administration (n=3) **(D)**, measured by fluorescence imaging system. Bars represent 1h timepoint (dark pink), 3h timepoint (light pink), 6h timepoint (dark green) or 24h timepoint (light green). Data represented as mean $\pm$ SEM of relative fluorescent signal (RFU/g of tissue).

In general, the ID administration route (**Figure 8B**) presented the lowest fluorescence values. After 1 hour of the injection, the fluorescence peaks ranged from 0 and 2246,59 RFU/grams of tissue with most of the fluorescence observed in PAT and stomach followed by axillary LN and bladder. After 3 hours, the lungs, MDLN, pancreas and thymus began to gather fluorescence and displayed maximum peaks at this timepoint, ranging from 0 to 2,282.11 RFU/g. At the 6-hour timepoint, fluorescence levels ranged between 0 and 1,775.83 RFU/g, with maximal peaks observed in organs such as the axillary LN and bladder. Finally, the 24-hour timepoint showed fluorescence values between 0.73 and 739.94 RFU/g, indicating a very slow biodistribution.

The IV administration route (**Figure 8C**) showed a rapid and systemic biodistribution among all the organs with fluorescence values ranging from 187.52 and 37,552 RFU/grams of tissue 1 hour after the administration. In general, the fluorescence was found to decrease progressively as time progressed. After 3 hours post-administration of DOPE-AF750-PS-liposomes, fluorescence levels were between 55.15 and 7,857.66 RFU/g. After 6 hours, fluorescence values ranged from 55.9 to 11,267.24 RFU/g and finally, the 24-hour timepoint showed a notable decrease of fluorescence in every organ with fluorescence levels between 7.34 and 2,422 RFU/g. Remarkably, this administration route demonstrated an enhanced delivery to the spleen and liver, organs consistently showing the highest peaks of fluorescence. Additionally, the lungs gathered considerable fluorescence levels, followed by some lymph nodes, including MDLN, PLN and axillary LN, as well as the bladder.

The IN-administration route (**Figure 8D**) revealed a restricted biodistribution profile since, lung and stomach exhibited the highest fluorescence peaks after the administration, along with some fluorescence observed in MDLN and axillary LN. One-hour post-administration of DOPE-AF750-PS-liposomes, fluorescence peaks ranged from 0 to 79,519.36 RFU/grams of tissue. Subsequently, as the time progressed the fluorescence values decreased, ranging from 0 to 67,463.33 RFU/g at 3-hour timepoint, and from 223.55 to 57,383.82 RFU/g after 6-hour timepoint. After

24 hours, maximum fluorescence peaks were notably decreased, with values ranging between 2.55 and 12,499.13 RFU/g.

3. NBD-PS-liposomes interact with immune cells in secondary lymphoid organs *in vivo*

Aiming to see which immune cells could interact *in vivo* with the NBD-labelled PS-liposomes, either empty (NBD-PS-empty-liposomes) or loaded with human insulin (NBD-PS-Ins-liposomes), prediabetic NOD mice were administered intraperitoneally, intradermally, or intravenously. IN administration was excluded due to its previously reported limited absorption. After 6 hours post-administration of a single dose of NBD-labelled PS-liposomes, organs of interest including the spleen, PLN and MDLN were collected and processed to obtain a single-cell suspension. Assessing which immune cell subsets were positive for the NBD fluorescence, we could investigate whether these cells were capable of interacting *in vivo* with the administered NBD-PS-liposomes. Flow cytometry gating strategy used in this test and the NBD positive immune cell subsets can be found in **Supplementary Figure 2**.

The cell viability after PS-liposomes administration in any of the different administration routes was optimal (**Supplementary Figure 3**). When mice were administered IP, ID or IV, splenocytes and cells from MDLN and PLN presented always presented a viability higher than $93.97 \pm 9.99\%$ (Mean $\pm$ SEM).

As expected, due to their well-documented capacity for efferocytosis, conventional dendritic cells (cDCs) (**Figure 9A**) exhibited one of the highest percentages of NBD positivity among immune cell subsets, with no significant differences observed between NBD-PS-Ins-liposomes and NBD-PS-empty-liposomes. In the spleen, the IP administration route presented the highest levels of NBD positivity, with for empty NBD-PS-liposomes $18.40 \pm 7.15\%$ and $14.47 \pm 9.73\%$ for insulin-loaded NBD-PS-liposomes. In contrast, the ID administration route, in comparison to IP route, showed significantly lower NBD positivity, with $0.37 \pm 0.62\%$ and $2.72 \pm 6.5\%$ for empty and loaded NBD-PS-liposomes, respectively ($p < 0.001$ and $p < 0.05$, respectively). IV administration route exhibited considerable high percentages of NBD positivity with both NBD-PS-empty-liposomes and NBD-PS-Ins-liposomes ($6.01 \pm 1.54\%$ and $11.29 \pm 7.88\%$). Although no differences were observed between loaded NBD-PS-liposomes, the empty group presented a statistically significant decrease when compared with the IP administration route ($p < 0.05$). MDLN cDCs showed a similar trend to those in the spleen; comparing the IP route, empty and loaded NBD-PS-liposomes had the highest positivity levels ($19.32 \pm 6.46\%$ and $17.06 \pm 7.74\%$) and

statistically higher than ID route ($1.63 \pm 2.75\%$ and $0.87 \pm 2.12\%$) ($p < 0.0001$ and $p < 0.001$, respectively). Regarding the IV administration route, NBD positivity levels did not show differences between empty or loaded groups ($5.89 \pm 5.11\%$ and $7.93 \pm 9.26\%$), although, NBD-PS-empty-liposomes showed a decrease in comparison to IP route ($p < 0.01$). Conversely, in PLN a different trend was observed since the cDCs after IV route exhibited the highest levels of NBD positivity ($11.39 \pm 7.01\%$ and $9.64 \pm 6.44\%$), statistically significantly higher than the ID route ($0.65 \pm 1.27\%$ and $0.86 \pm 1.53\%$) ($p < 0.01$ and $p < 0.01$). In the IP route, cDCs showed slightly decreased levels of NBD positivity in both, empty and loaded PS-NBD-liposomes ($5.21 \pm 3.80\%$ and $4.91 \pm 1.53\%$), although no statistical significance.

Plasmacytoid dendritic cells (pDCs) (**Figure 9B**), in general, presented lower NBD positivity levels than cDCs. In the spleen, after the IP administration of NBD-PS-empty-liposomes and NBD-PS-Ins-liposomes, pDCs presented the highest levels of positivity, $8.05 \pm 4.81\%$ and $8.88 \pm 8.10\%$, respectively. In comparison ID presented statistically significant lower levels of NBD positivity in both conditions ($1.14 \pm 0.96\%$ and $1.91 \pm 3.01\%$) ($p < 0.05$ and $p < 0.05$). Percentages of NBD after IV presented no differences between empty or loaded PS-liposomes ($1.30 \pm 1.06\%$ and $3.60 \pm 2.95\%$), although NBD-PS-empty-liposome in comparison to IP showed a statistically significant decrease ($p < 0.05$). In MDLN, IP and IV administration routes presented similar NBD positivity values either with empty or loaded NBD-PS-liposomes ($6.97 \pm 2.79\%$ and $6.08 \pm 2.97\%$) ($5.28 \pm 4.09\%$ and $7.00 \pm 5.29\%$), respectively. In contrast, the ID route showed a statistically significant decrease when comparing NBD-PS-Ins-liposomes with IP and IV routes ($2.83 \pm 2.50\%$ and $1.15 \pm 1.92\%$) ($p < 0.05$ and $p < 0.05$). PLN pDCs, presented the highest NBD levels when, both empty or loaded, PS-liposomes were administered IV ($5.84 \pm 0.10\%$ and $9.91 \pm 5.22\%$), being statistically increased in comparison to NBD-PS-Ins-liposomes administered ID ($2.60 \pm 2.01\%$) ($p < 0.05$) and IP ($3.72 \pm 4.17\%$) ($p < 0.05$).

M ϕ (**Figure 9C**) were the immune cell subset that showed the highest NBD positivity percentages together with cDCs. No differences were observed between NBD-PS-Ins-liposomes and NBD-PS-empty-liposomes in any administration route nor organ. Nevertheless, the IP administration route always presented higher NBD percentages, in spleen, empty and loaded conditions presented a positivity of $30.12 \pm 22.44\%$ and $28.26 \pm 19.78\%$, respectively, being statistically significant higher than the ID route ($1.48 \pm 3.36\%$ and $1.18 \pm 2.23\%$) ($p < 0.01$ and $p < 0.01$) and IV route only with NBD-PS-empty-liposomes ($5.19 \pm 3.34\%$) ($p = 0.05$). M ϕ from MDLN presented the same trend, where IP levels ($20.92 \pm 16.68\%$ and $24.32 \pm 18.21\%$) were statistically significant higher in comparison to ID route ($0.40 \pm 0.98\%$ and $1.67 \pm 2.64\%$) ($p < 0.01$ and $p < 0.01$).

and IV route ($4.19 \pm 2.21\%$ and $6.33 \pm 0.80\%$) ($p < 0.05$ and $p < 0.05$). Remarkably, PLN M ϕ showed the highest NBD positivity levels, being after the i.p injection with empty and loaded PS-NBD-liposomes $57.33 \pm 16.68\%$ and $67.86 \pm 33.79\%$, statistically increased in comparison to ID route ($13.72 \pm 32.42\%$ and $15.18 \pm 37.19\%$) ($p < 0.05$ and $p < 0.01$) and IV route ($3.17 \pm 1.17\%$ and $2.17 \pm 1.6\%$) ($p < 0.05$ and $p < 0.01$).

Surprisingly, the B cell subset presented some NBD positivity (**Figure 9D**), particularly with the IP administration route, indicating an interaction between a small percentage of B cells and PS-NBD-liposomes. After IP injection, with NBD-PS-empty-liposomes and NBD-PS-Ins-liposomes, B cells in the spleen showed positivity percentages of $1.50 \pm 0.90\%$ and $1.36 \pm 1.20\%$, respectively. These levels were statistically increased in comparison to the ID route ($0.03 \pm 0.06\%$ and $0.17 \pm 0.40\%$) ($p < 0.01$ and $p < 0.05$) and the empty condition with IV route ($0.44 \pm 0.37\%$ and $0.91 \pm 0.64\%$) ($p < 0.05$). MDLN B cells presented the same trend, IP administration route showed elevated NBD positivity values of $3.20 \pm 1.11\%$ with NBD-PS-empty-liposomes and $2.99 \pm 1.48\%$ with NBD-PS-Ins-liposomes, being statistically significant higher in comparison to ID route ($0.51 \pm 1.11\%$ and $0.07 \pm 0.11\%$) and to IV route ($0.33 \pm 0.13\%$ and $0.33 \pm 0.15\%$) presenting a p value always < 0.0001 . The same trend was observed in PLN B cells, presenting the highest NBD positivity percentage with the IP administration ($1.36 \pm 1.20\%$ and $1.50 \pm 0.90\%$) and statistically increased when compared to ID and IV administration routes ($0.17 \pm 0.40\%$ and $0.03 \pm 0.06\%$) ($p < 0.01$) and ($0.44 \pm 0.37\%$ and $0.91 \pm 0.64\%$) ($p = 0.05$ with NBD-PS-empty-liposomes), respectively.

Overall, regardless of the administration route, T cells exhibited minimal interaction with the fluorescently labelled PS-liposomes, with NBD positivity always less than 1% of the total T cell subset (**Figure 9E**). T cells from the spleen presented the highest interaction when empty or loaded NBD-PS-liposomes were injected IP ($1.00 \pm 0.78\%$ and $0.89 \pm 0.75\%$), being statistically significant higher than ID administration route (0.02 ± 0.03 and $0.15 \pm 0.35\%$) ($p < 0.01$ and $p < 0.05$) and IV ($0.08 \pm 0.09\%$ and $0.35 \pm 0.37\%$) ($p < 0.01$ with NBD-PS-empty-liposomes). MDLN T cells showed the same trend, IP NBD positivity levels after empty or loaded NBD-PS-liposome administration were 0.43 ± 0.3 and $0.54 \pm 0.37\%$, statistically significant higher than ID administration route ($0.14 \pm 0.30\%$ and $0.004 \pm 0.01\%$) ($p < 0.01$ with NBD-PS-Ins-liposomes) and IV route ($0.003 \pm 0.01\%$ and $0.006 \pm 0.004\%$) ($p < 0.05$ and $p < 0.001$). Although the levels of T cell positivity in the PLN were almost equal to zero; after intraperitoneally administering NBD-liposomes, the levels of positivity were statistically significant higher ($0.63 \pm 0.35\%$ and $0.14 \pm 0.11\%$) than those obtained

RESULTS

with the ID ($0.03 \pm 0.06\%$ and $0.02 \pm 0.02\%$) ($p < 0.05$ and $p < 0.01$) and IV routes ($0.01 \pm 0.01\%$ and $0.01 \pm 0.01\%$) ($p < 0.01$ with NBD-PS-empty-liposomes).

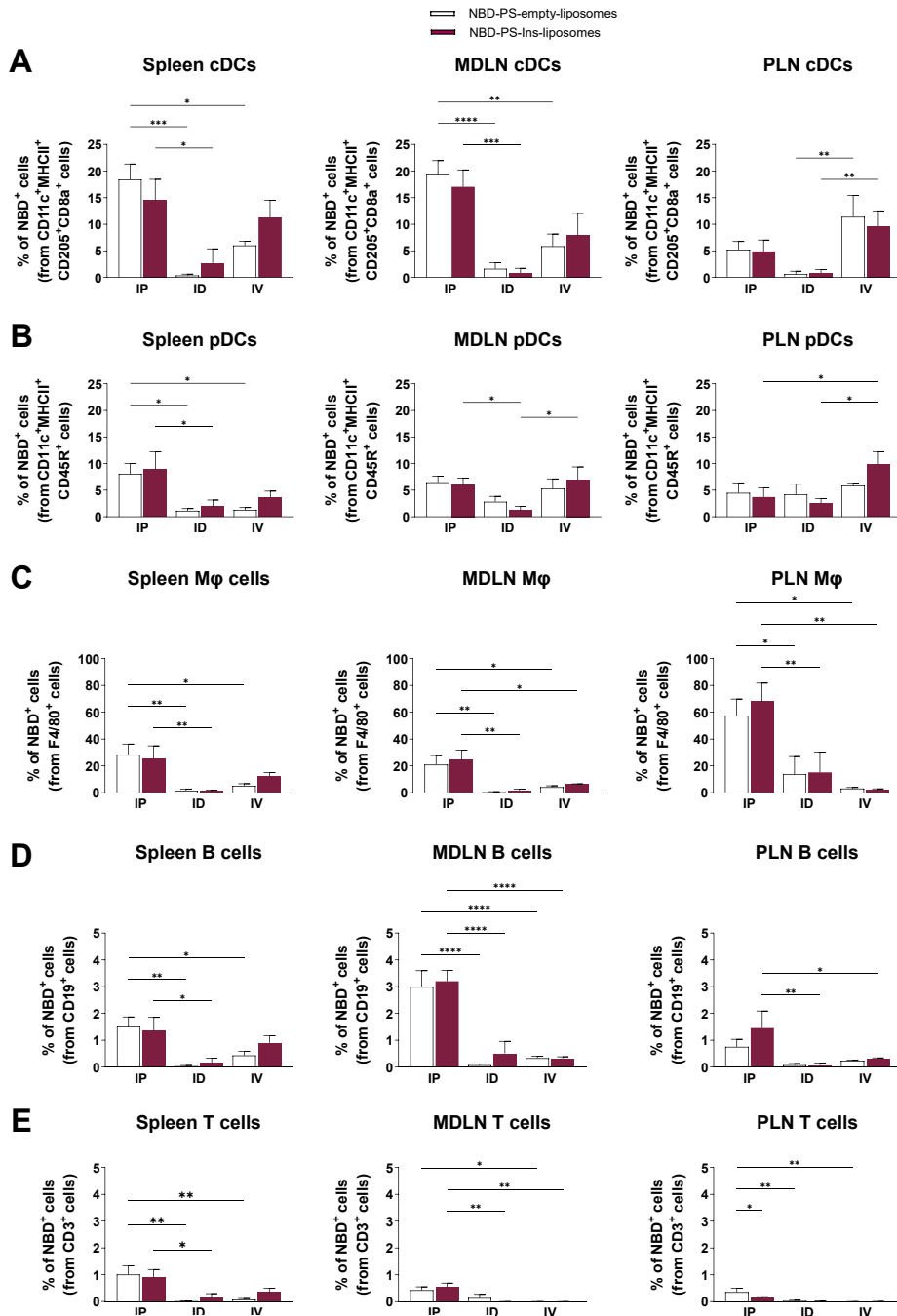


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Figure 9. Percentages of NBD-fluorescent cells of the parent gate in the spleen of NOD mice after 6h of the NBD-labelled PS-liposomes intraperitoneally, intradermally or intravenously administration (n=5-6), measured by flow cytometry. (A) Conventional Dendritic Cells subset (cDCs), (B) plasmacytoid Dendritic Cell subset (pDCs), (C) Macrophages subset (Mφ), (D) B cell subset, (E) T cell subset. Bars represent NBD-PS-empty-liposomes (white) and NBD-PS-Ins-liposomes (dark pink). Data represented as mean $\pm$ SEM. Significant differences were found when comparing different administration routes (****p< 0.0001, ***p< 0.001, **p<0.01, *p $\leq$ 0.05, two-way ANOVA with Tukey's multiple comparison test).

4. IV administration route directs the interaction *in vivo* of PS-Ins-liposomes with phagocytic immune cells in target organs

The mononuclear phagocyte (MPS) or reticuloendothelial (RES) system are terms widely used in the nanomedicine field to refer to cells that represent a biological barrier to NP delivery. RES term encompasses various endothelial cell types that exhibit endocytic behaviours, while MPS refers to many of the cells involved in clearing macromolecules and foreign particulates from the blood such as monocytes or cells originated from bone marrow [400,401]. Both are present in various organs, including the spleen and liver. One of the primary functions of this system is the sequestration and clearance of materials present in the blood. Given their implication in the peripheral tolerance induction and the high signal exhibited after the IV administration, the liver and the spleen were further studied to determine which regulatory immune cells could interact *in vivo* with the PS-liposomes. To achieve this and having seen that no differences between empty or loaded PS-liposomes existed, NBD-labelled PS-Ins-liposomes were administered into prediabetic NOD mice. Given that the IV administration route demonstrated a faster delivery compared to the other studied routes, fluorescence was assessed in the spleen and liver after 15 minutes, 1- or 6 hours post-injection. Flow cytometry gating strategy used in this test and the NBD positive immune cell subsets can be found in **Supplementary Figure 4 and 5**.

Splenocytes and liver cells showed always a high viability around, $96.34 \pm 0.88\%$ and $96.96 \pm 1.70\%$, respectively (Mean $\pm$ SD, **Supplementary Figure 6**). Regarding the spleen (**Figure 10A**), the highest NBD positivity levels were generally detected across all immune subsets 15 minutes post-administration. Mφ showed the highest percentage of NBD positivity, $32.99 \pm 13.58\%$ after 15 minutes. At the 1-hour time point, there was a statistically significant decrease in NBD positivity ($20.68 \pm 8.58\%$) ($p<0.01$) and this trend continued at the 6-hour timepoint decreasing until $17.79 \pm 6.19\%$ of positivity ($p<0.0001$). The DC general subset showed similar capture

dynamics, with NBD fluorescence reaching percentages of 15.14 ± 4.67 after 15 minutes of administration followed by a significant decrease at the 1-hour timepoint ($6.03 \pm 2.29\%$) ($p < 0.05$) and a further reduction at the 6-hour timepoint ($4.76 \pm 1.75\%$) ($p < 0.01$). Within the different subpopulations of DCs, it was found that cDCs presented higher phagocytosis rates than pDCs, specially cDC1s which presented the highest peak at 15 minutes post-injection ($18.78 \pm 6.66\%$). Although not statistically significant, NBD positivity decreased progressively over time, with levels at the 1-hour timepoint of $15.83 \pm 5.28\%$ and finally at the 6-hour timepoint of $10.00 \pm 5.63\%$. CDC2s exhibited less NBD fluorescence, with levels after 15 minutes reaching percentages of 7.73 ± 3.84 . However, 1 hour after the administration levels of NBD positivity were maintained ($7.73 \pm 3.52\%$) and started to slightly decrease at the 6-hour timepoint ($5.57 \pm 5.44\%$). PDCs presented a remarkably elevated peak after 15 minutes post-administration ($14.88 \pm 4.29\%$). Nevertheless, the NBD signal significantly decreased at the 1h post-injection timepoint ($3.71 \pm 4.50\%$) ($p < 0.05$) and remained significantly lower at the 6-hour timepoint ($4.79 \pm 1.47\%$) ($p < 0.01$). On this occasion, non-phagocytic cells such as B and T cell subsets presented barely any interaction, with NBD percentage positivity less than 1%.

In the liver (**Figure 10B**), the overall capture percentages were higher than those observed in the spleen at all time points studied. The highest levels of NBD positivity were observed in Kupffer cells, which presented levels of $97.12 \pm 0.80\%$ just after 15 minutes post-administration. Although not statistically significance, the NBD signal decreased to $71.40 \pm 17.04\%$ after 1 hour and remained constant after 6 hours ($75.17 \pm 20.84\%$). The same pattern of capture was observed for all cDCs, with NBD positivity levels of $35.93 \pm 2.35\%$ for cDC1s and $31.59 \pm 4.06\%$ for cDC2s observed after 15 minutes. Subsequently, at 1-hour post-administration, cDCs showed their highest peak, reaching $42.80 \pm 5.41\%$ and $42.35 \pm 6.38\%$ for cDC1c and cDC2s, respectively. These values were found to be statistically higher compared to the previous time point ($p < 0.05$ with cDC1s). However, after 6 hours, the NBD positivity values considerably decreased to $15.93 \pm 8.31\%$ and $14.95 \pm 8.13\%$, respectively. These values were statistically lower compared to those observed at the 1-hour time point ($p < 0.05$ and $p < 0.05$). LSECs also presented notably high NBD levels after 15 minutes reaching percentages of NBD positivity of 33.41 ± 4.59 . Although not statistically significance, the NBD signal decreased over time, with levels at the 1-hour timepoint of $25.05 \pm 13.16\%$ and finally at the 6-hour timepoint of $15.10 \pm 11.83\%$. Non-phagocytic cells consistently presented low levels of NBD positivity. Nevertheless, the B cell subset, although non-significant, showed its highest peak at a 1-hour timepoint

reaching $12.89 \pm 9.74\%$. Similarly, T cells showed the same trend with values reaching $6.25 \pm 0.07\%$ after 1-hour post-administration.

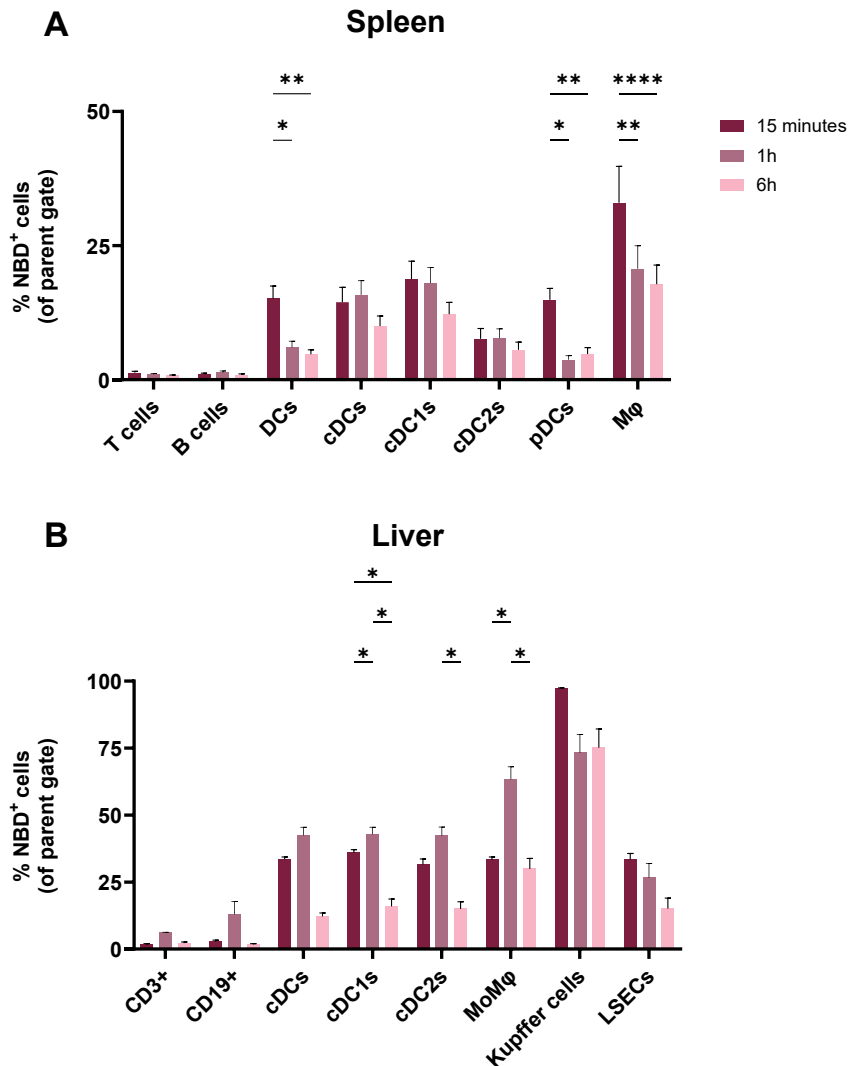


Figure 10. Percentages of NBD-fluorescent cells of the parent gate in the target organs of NOD mice after 15 minutes, 1- or 6-hours of the NBD labelled PS-liposomes intravenously administration, measured by flow cytometry. (A) Immune cell subsets in the spleen (n=4-9), (B) Immune cell subsets in the liver (n=4-9). Bars represent a 15-minute timepoint (dark pink), 1h timepoint (mid pink), and 6h timepoint (light pink). Data represented as mean $\pm$ SEM. Significant differences were found when comparing different administration routes (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, Mixed-effects model (REML) with Tukey's multiple comparison test).

5. PS-AChR-liposomes administered intravenously into C57BL/6 mice reach organs of interest

At this point, three administration routes—IP, IV, and ID— were considered for future clinical trials in humans. Nevertheless, based on the previous results, IV administration route was selected as the most appropriate for human application due to its efficacy and targeted distribution to APCs located in the spleen and liver. The ID administration route was discarded due to the low levels of fluorescence observed in organs of interest during the biodistribution experiments and the low interaction between PS-liposomes and immune cell subsets. Despite IP administration route also demonstrated high fluorescence values in target organs and efficient interaction with APCs, ultimately, was not selected due to the technical difficulties and invasive nature of the route. Given that regulatory guidelines issued by multiple regulatory agencies require a comprehensive biodistribution study, the next natural step was to better assess the biodistribution of the selected IV administration route. Considering Ahead Therapeutic's leading asset, MG disease, the extensive biodistribution experiment was performed with the correspondent PS-AChR-liposomes. This time, instead of using NOD mice, the murine model of T1D, the mice strain C57BL/6 was used since it is extensively used for inducing experimental autoimmune MG [402].

To that end, DOPE-AF750-labelled PS-AChR-liposomes were intravenously administered to male and female C57BL/6 mice and organs of interest were assessed for fluorescence in a time course ranging from 15 minutes to 48 hours (**Figure 11**). 15 minutes after the injection, IV administration demonstrated a rapid biodistribution, evidenced by high fluorescence peaks in most of the organs, which ranged from 1,647.18 to 51,280.90 RFU/gram of tissue. Over time, generally, the fluorescence levels in these organs decreased to levels between 1,132.42 and 29,361.76 RFU/g after 1h (**Supplementary Figure 7**). Nevertheless, certain organs including the spleen, mesenteric LN, PLN, axillary LN, salivary LN, stomach and hind limb bones presented their highest fluorescence peaks at the 1-hour timepoint, ranging from 3836.05 to 80,907.50 RFU/g (**Supplementary Figure 11A**). At the 6-hour timepoint, fluorescence peaks significantly decreased to levels between 156.70 and 15,753.75 RFU/g. Notably, the bladder was the only organ showing its highest peak at this timepoint (7,824.26 RFU/g) (**Supplementary Figure 11B**), suggesting the bladder as the primary secretory pathway for PS-liposomes. As expected, due to the biodegradability nature of PS-liposomes, the signal in almost every organ was null at 24- and 48-hour timepoint post-administration, demonstrating no bioaccumulation with very low

fluorescence levels ranging from 12.83 to 2,224.41 RFU/g, and from 2.73 to 1,249.84 RFU/g, respectively.

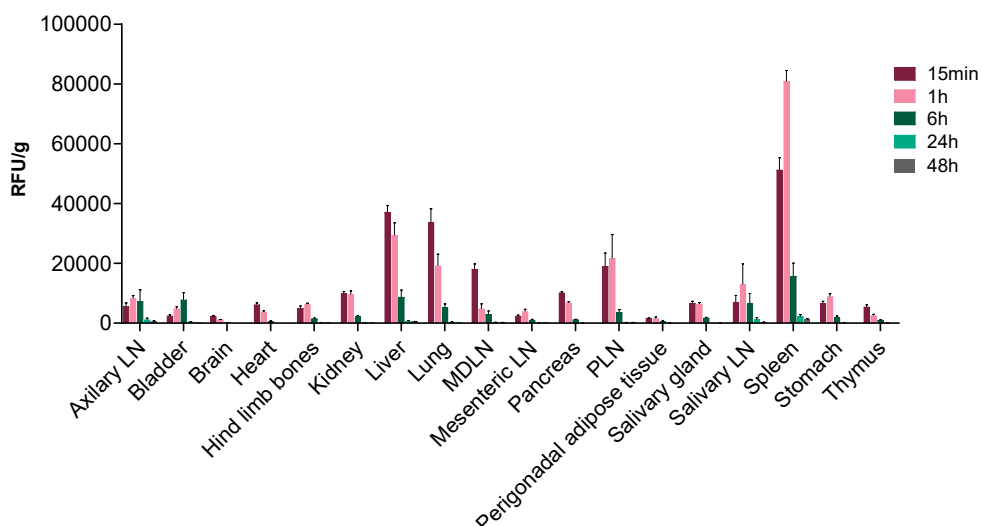


Figure 11. PS-AF750-AChR-liposomes biodistribution after IV administration of AF750-PS-AChR-liposomes in C57BL/6 mice (n=3-6). Bars represent fluorescence intensity at 15 minutes timepoint (dark pink), 1h timepoint (light pink), 6h timepoint (dark green), 24h timepoint (light green) and 48h timepoint (grey), measured by a fluorescent imaging system. Data represented as mean $\pm$ SEM of relative fluorescent signal (RFU/g of tissue).

6. Intravenously administered NBD-labelled PS-AChR-liposomes are broadly *in vivo* engulfed by phagocytes in target organs and by circulating immune cells

Until now, we have seen that PS-liposomes administered intravenously are rapidly distributed to organs with vascular supply, where they are rapidly captured and can persist, maintaining high levels of interaction with phagocytic cells. After 1h of the administration, PS-liposomes start to be cleared from the system, via the renal, liver or reticuloendothelial system. To better evaluate the PS-liposomes uptake dynamics in the target organs, it was performed an extensive *in vivo* study with the fluorescent NBD-labelled PS-AChR-liposomes. Male and female C57BL/6 mice were intravenously injected with NBD-PS-AChR-liposomes. After 15 minutes, 1 hour, 6

hours, 24 hours, or 48 hours post-injection, organs of interest, including the spleen and liver, were harvested for analysis. Blood samples were also collected at each time point to investigate potential interactions with circulating immune cell subsets. An improved strategy gating was applied and optimised for each organ (**Supplementary Figure 9-11**).

Consistent with previous *in vivo* experiments using NOD mice, this time splenocytes from C57BL/6 mice showed that DCs and M ϕ were the immune cell subsets exhibiting the highest percentages of NBD positivity in this study, specifically the cDCs1 followed by the red pulp M ϕ , monocyte-derived macrophages (MoM ϕ) and white pulp/ marginal zone M ϕ (**Supplementary Figure 12**). In contrast, a general subset of DCs showed the maximum fluorescence peak after 1h of IV administration of NBD-PS-AChR-liposomes, in the case of cDCs NBD percentages significantly increased from 10.86 ± 2.51 (Mean $\pm$ SD) at 15 minutes to 14.82 ± 3.13 ($p < 0.05$) (**Figure 12**). At the 6-hour timepoint, the NBD positivity decreased significantly to levels of $2.52 \pm 0.59\%$ ($p < 0.0001$), and at 24- and 48-hours NBD decreased drastically to $0.02 \pm 0.04\%$ ($p < 0.0001$) and $0.05 \pm 0.09\%$ ($p < 0.0001$), respectively. Although cDCs1 exhibited higher NBD-PS-AChR-liposomes uptake than cDCs2, the two subtypes followed the same pattern, both showing the maximum NBD fluorescence peak after 1h of administration. Specifically, NBD positivity increased from $30.52 \pm 3.70\%$ to $37.92 \pm 8.61\%$ in cDCs1 and from $7.78 \pm 2.44\%$ to $10.61 \pm 2.53\%$ in cDC2 cells. At the 6-hour time point, a significant reduction in NBD fluorescence was observed, with levels decreasing to $8.46 \pm 2.81\%$ ($p < 0.0001$) in cDCs1 and $0.63 \pm 0.25\%$ ($p < 0.0001$) in cDC2 cells. Fluorescence at the 24- and 48-hour time points was barely detectable in any of the DC subtypes. PDCs also presented the highest NBD fluorescence level after 1h of administration, reaching percentages of 8.52 ± 3.44 and decreasing significantly after 6 hours with values of 2.33 ± 1.84 ($p < 0.01$). After 24- and 48-hours timepoints NBD percentages continued to decline, reaching levels close to zero. Monocytes in the spleen did not reach high rates of phagocytosis, presenting NBD percentages of 2.08 ± 0.97 after 15 minutes and remaining stable after 1h ($2.55 \pm 2.32\%$). NBD positivity levels started to decrease at 6 h (1.12 ± 0.39) and became more pronounced at 24 and 48h, being nearly undetectable. Conversely, moM ϕ displayed a different capture pattern, emerging as the third subset with the highest NBD percentages and showing the peak NBD-PS-AChR-liposome uptake almost immediately after administration ($18.14 \pm 5.40\%$). Over time, NBD percentages decreased slightly after 1 hour to $13.37 \pm 5.75\%$, followed by a more pronounced decline at 6 hours to $5.34 \pm 2.52\%$ ($p < 0.01$), eventually reaching levels below 1% at 24 and 48 hours ($p < 0.0001$). Red pulp M ϕ

were the second immune cell subset with the highest percentage of fluorescent liposome capture. Following the same pattern as the MoM ϕ , they showed the highest percentage of NBD immediately after administration (30.89 ± 9.45) and over time, this percentage decreased progressively, with a reduction to $28.53 \pm 13.45\%$ after 1 hour. A significant decrease was observed starting at 6 hours, with values of $12.16 \pm 5.48\%$ ($p < 0.01$), followed by further decreases to $1.47 \pm 0.68\%$ ($p < 0.0001$) after 24 hours and $1.03 \pm 0.63\%$ ($p < 0.0001$) after 48 hours, compared to the 15 minutes timepoint. White pulp and marginal zone M ϕ although having lower percentages, presented the same uptake pattern, with higher NBD percentages just after the IV administration of the PS-liposomes ($16.59 \pm 3.29\%$). This positivity began to decrease slightly after 1 hour to $14.84 \pm 6.07\%$ and showed a significant reduction after 6 hours to $4.18 \pm 0.88\%$ ($p < 0.0001$). This reduction persisted, with levels decreasing to $1.15 \pm 0.36\%$ ($p < 0.0001$) after 24 hours and reaching $0.78 \pm 0.38\%$ by 48 hours. Finally, B and T cells presented a slight interaction with NBD-labelled PS-AChR-liposomes reaching their highest NBD percentages 1h after the administration ($0.96 \pm 0.74\%$ and $1.44 \pm 0.56\%$), respectively. At 6h, like the rest of the subpopulations, they started to decrease significantly, $0.18 \pm 0.08\%$ ($p < 0.05$) and $0.45 \pm 0.23\%$ ($p < 0.0001$) until they were barely undetectable after 24- and 48-hours.

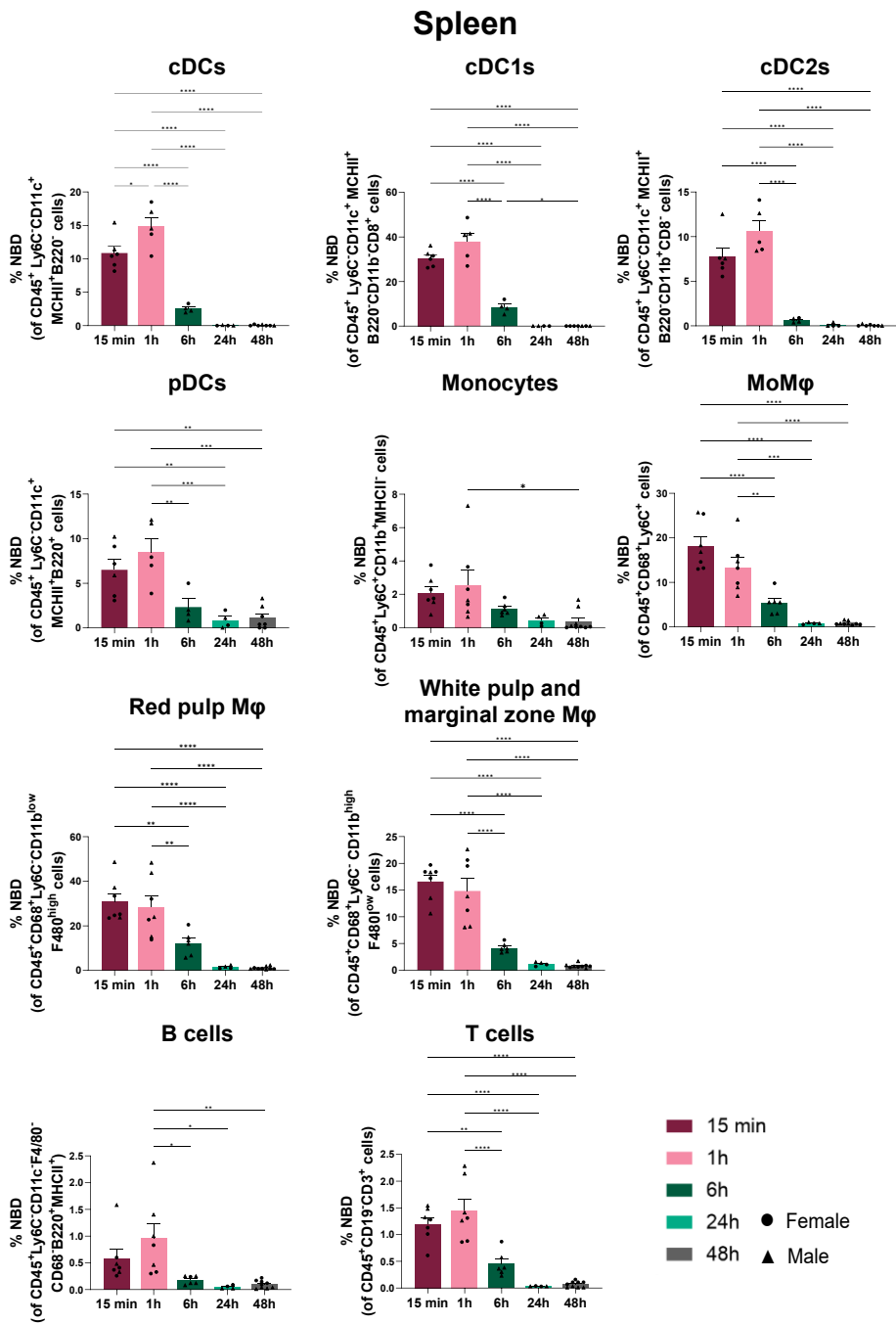


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Figure 12. Percentages of NBD-fluorescent leukocytes of the parent gate in the spleen of C57BL/6 mice after the NBD-labelled PS-AChR-liposomes IV administration, measured by flow cytometry (n=4-7). 15-minute timepoint (dark pink), 1h timepoint (light pink), 6h timepoint (dark green), 24h timepoint (light green) and 48h timepoint (grey). Each black dot represents a female mouse, and each black triangle represents a male mouse. Data represented as mean $\pm$ SEM. Significant differences were found when comparing different time points (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, one-way ANOVA with Tukey's multiple comparison test).

Phagocytosis of NBD-PS-AChR-liposomes by liver immune cell subsets was consistent with previous findings in NOD mice. Kupffer cells exhibited the greatest capacity for liposome capture, followed by LSECs and MoM ϕ (**Supplementary Figure 13**). CDCs maintained the same dynamic pattern of capture, with NBD positivity reaching $9.26 \pm 1.47\%$ at 15 minutes and significantly increasing to a peak of $14.92 \pm 5.56\%$ ($p < 0.05$) after 1 hour of the administration of the fluorescent PS-AChR-liposomes (**Figure 13**). As expected, a pronounced decrease was observed at the 6-hour timepoint, dropping to $3.56 \pm 1.07\%$ ($p < 0.0001$). This decrease became even more pronounced at 24 and 48 hours with values of $0.47 \pm 0.44\%$ and $0.56 \pm 0.43\%$ respectively. Both cDCs1 and cDCs2 followed the same pattern and showed similar PS-liposome uptake percentages, after 15 minutes of administration, the NBD positivity in these cells increased from $14.75 \pm 5.02\%$ and $10.79 \pm 2.47\%$, respectively, to 17.45 ± 4.60 and 12.48 ± 2.81 percentages, respectively, after 1h. The significant decrease occurred after 6h of NBD-PS-AChR-liposomes administration with values of $5.60 \pm 1.61\%$ ($p < 0.0001$) and $1.70 \pm 0.74\%$ ($p < 0.0001$), and fluorescence continued to diminish at 24 and 48 hours until NBD levels were undetectable. pDCs after 15 minutes exhibited relatively low phagocytosis levels ($10.37 \pm 9.37\%$), however, after 1h the percentage significantly increased to 42.06 ± 23.17 ($p < 0.01$). By the 6-hour timepoint, NBD positivity began to decline ($21.93 \pm 9.24\%$), and by 24 and 48 hours it decreased significantly to levels approaching zero. Liver monocytes, however, exhibited similar NBD positivity levels at 15 minutes and 1-hour post-administration, with values of $15.99 \pm 5.18\%$ and $16.76 \pm 8.90\%$, respectively. The percentage of NBD positivity started to decrease after 6h, reaching $5.13 \pm 1.69\%$ ($p < 0.001$). By the 24- and 48-hour time points, percentages of NBD fell by below 1%, 0.86 ± 0.73 ($p < 0.0001$) and 0.63 ± 0.24 ($p < 0.0001$). MoM ϕ , as previously seen in the spleen, presented faster capture dynamics, with their highest NBD positivity peak only after 15 minutes of the administration, $27.61 \pm 5.18\%$. Over time, the percentage began to decrease significantly, dropping to values of $18.53 \pm 3.25\%$ ($p < 0.01$) after 1 hour and $7.45 \pm 3.25\%$ ($p < 0.001$) after 6 hours. By 24- and 48-hour time points percentages decreased to levels of 1.10 ± 0.36 and 1.58 ± 0.18 , respectively. Kupffer cells, the specialized M ϕ

in the liver, demonstrated high percentages of NBD-PS-AChR-liposome capture as early as 15 minutes post-administration, reaching levels of $86.14 \pm 12.72\%$, after 1h, NBD positivity peaked with percentages of 94.14 ± 2.89 . Notably, unlike other liver immune cell subsets, Kupffer cells maintained high NBD positivity levels ($84.86 \pm 12.72\%$) even after 6 hours. Nevertheless, by the 24- and 48-hour time points, these percentages of capture decreased significantly to $5.22 \pm 4.69\%$ ($p < 0.0001$) and $1.96 \pm 1.31\%$ ($p < 0.0001$), respectively. This time, LSECs presented high percentages of capture, with the peak of NBD observed at 15 minutes ($72.50 \pm 6.01\%$). As time progressed, these percentages gradually decreased with values of $51.71 \pm 12.70\%$ after 1 hour followed by a notably significant decrease at 6 hours ($5.85 \pm 2.67\%$) ($p < 0.0001$). NBD positivity further decreased finally to $1.46 \pm 0.78\%$ ($p < 0.0001$) after 24 hours and $1.56 \pm 1.02\%$ ($p < 0.0001$) after 48 hours. Lastly, B and T cells exhibited a relatively high interaction with NBD-labelled PS-AChR-liposomes, reaching their highest NBD positivity percentages 1 hour after administration, with $5.46 \pm 6.26\%$ and $4.98 \pm 3.63\%$, respectively. By 6 hours, similarly to other subpopulations, these percentages decreased, dropping to $0.20 \pm 0.12\%$ and $1.73 \pm 2.08\%$.

Liver

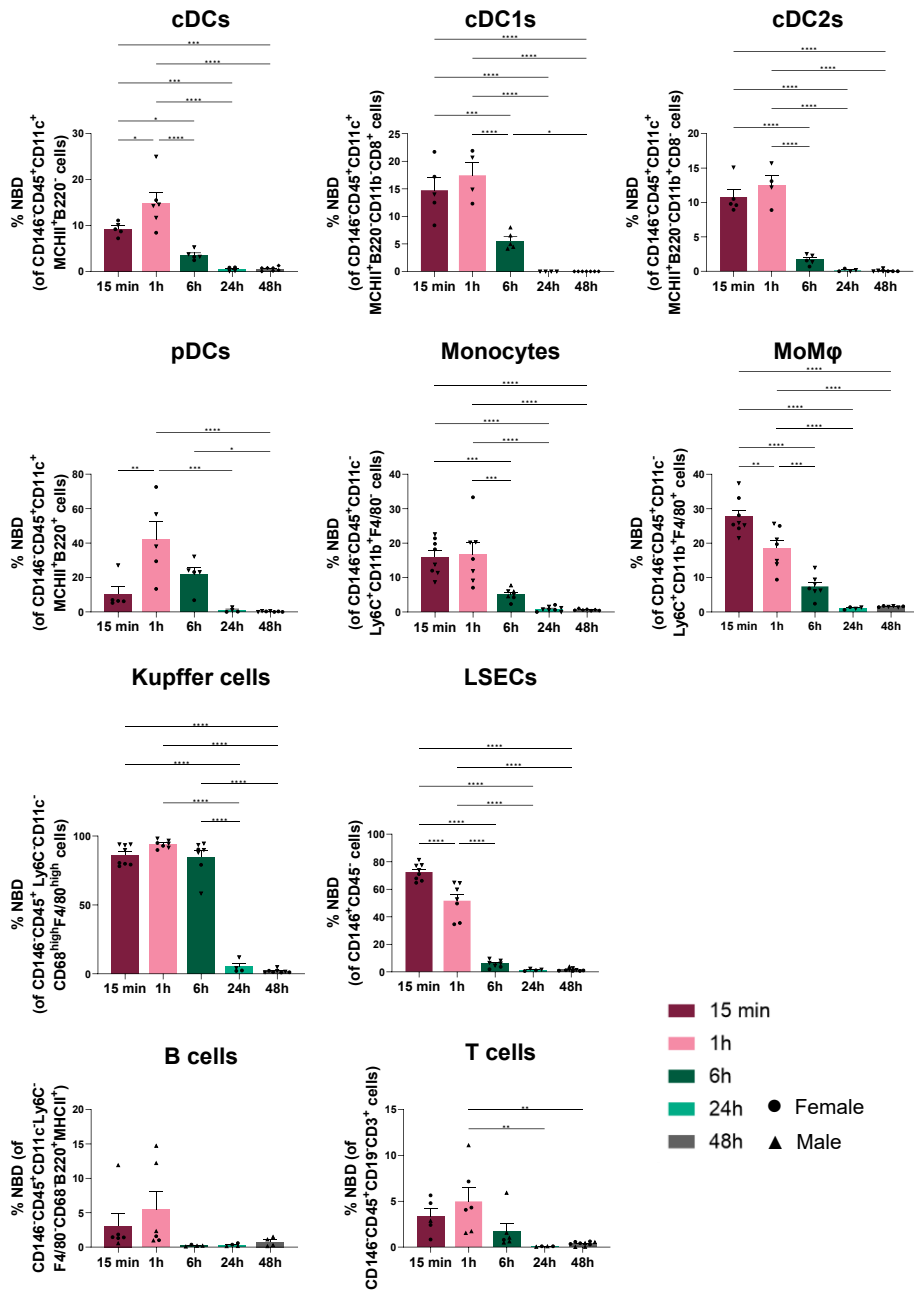


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Figure 13. Percentages of NBD-fluorescent leukocytes of the parent gate in the liver of C57BL/6 mice after the NBD-labelled PS-AChR-liposomes IV administration, measured by flow cytometry (n=4-7). 15-minute timepoint (dark pink), 1h timepoint (light pink), 6h timepoint (dark green), 24h timepoint (light green) and 48h timepoint (grey). Each black dot represents a female mouse, and each black triangle represents a male mouse. Data represented as mean $\pm$ SEM. Significant differences were found when comparing different time points (****p< 0.0001, ***p< 0.001, **p<0.01, *p $\leq$ 0.05, one-way ANOVA with Tukey's multiple comparison test).

For the first time, the circulating immune cell subsets were evaluated after the NBD-PS-AChR IV administration, although interaction with circulating immune cells is difficult due to the continuous circulation through the circulatory system. Noteworthy, circulating monocytes were the immune cell subset showing the highest ability to interact *in vivo* with PS-AChR-liposomes, followed by M ϕ and neutrophils (**Supplementary Figures 14**). Circulating cDCs presented some ability to capture NBD-labelled PS-AChR-liposomes 15 minutes after the administration with a positivity percentage of 3.45 ± 5.99 (**Figure 14**). However, this percentage immediately decreased to below 1% after 1h of injection ($0.24 \pm 0.32\%$). Notably, only cDCs1 subset showed positivity for NBD-PS-AChR-liposomes at the 15-minute timepoint ($2.22 \pm 4.12\%$), while NBD positivity in cDCs2 always remained undetectable (data not shown). For the plasmacytoid pDC subset, NBD positivity was detected at $2.98 \pm 4.77\%$ 15 minutes after administration. This percentage gradually decreased to $1.78 \pm 1.58\%$ at the 1-hour timepoint and fell below 1% by the 6-hour timepoint ($0.93 \pm 0.22\%$). Monocytes exhibited a remarkably high NBD percentage immediately after the PS-AChR-liposomes ($72.01 \pm 9.35\%$). However, this percentage decreased significantly after 1 hour, dropping to $2.45 \pm 2.73\%$ (p<0.0001), and being almost undetectable at a 6-hour timepoint ($0.21 \pm 0.1\%$) (p<0.0001). Although circulating M ϕ were present in low numbers (data not shown), they demonstrated some capacity to capture PS-AChR-liposomes as early as 15 minutes post-injection with NBD positivity percentages of $7.83 \pm 6.28\%$. After 1 hour, this percentage started to decrease gradually ($0.90 \pm 0.59\%$) (p<0.01) and became nearly undetectable after 6 hours, reaching ($0.37 \pm 0.29\%$) (p<0.001). Neutrophils, recognized for their phagocytic function, exhibited NBD positivity 15 minutes post-administration, with levels reaching $6.01 \pm 5.03\%$. These levels decreased after 1 hour ($2.95 \pm 1.65\%$) and were further reduced at the 6-hour timepoint ($1.73 \pm 0.73\%$). Interestingly, NBD positivity remained relatively stable at 24 and 48 hours, with levels of $2.43 \pm 0.41\%$ and $2.25 \pm 1.35\%$, respectively. Finally, B and T cells showed similar NBD positivity values consistently below 1. At 15 minutes post-administration, the NBD percentages were $0.28 \pm 0.14\%$ and $0.32 \pm 0.23\%$, respectively. Notably, B cells reached their

maximum interaction peak at 1 hour ($0.37 \pm 0.17\%$), after which the levels began to significantly decrease, eventually becoming undetectable.

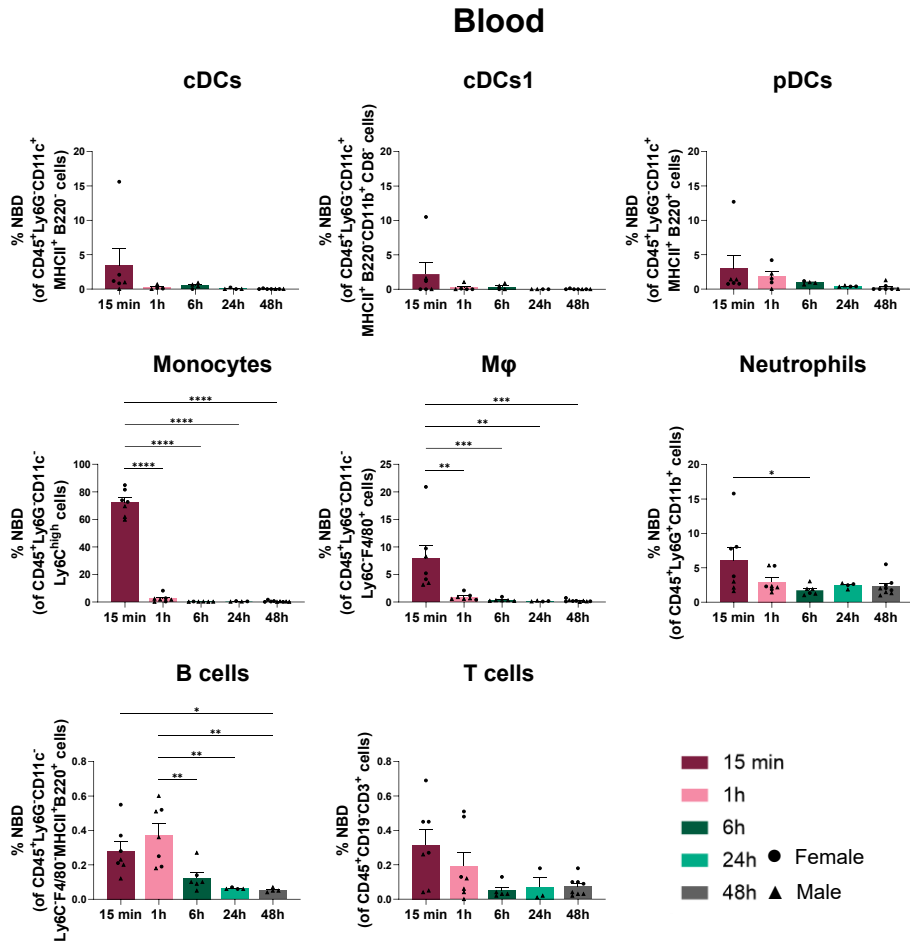


Figure 14. Percentages of NBD-fluorescent cells of the parent gate in the circulating blood of C57BL/6 mice after the NBD-labelled PS-AChR-liposomes IV administration, measured by flow cytometry (n=4-7). 15-minute timepoint (dark pink), 1h timepoint (light pink), 6h timepoint (dark green), 24h timepoint (light green) and 48h timepoint (grey). Each black dot represents a female mouse, and each black triangle represents a male mouse. Data represented as mean $\pm$ SEM. Significant differences were found when comparing different time points (****p< 0.0001, ***p< 0.001, **p<0.01, *p $\leq$ 0.05, one-way ANOVA with Tukey's multiple comparison test).

SECTION II. Antigen-specific immunomodulation of loaded PS-liposomes

Antigen-specific immunotherapies represent a key approach in autoimmune disease treatment by selectively targeting pathogenic immune responses while preserving protective immunity. Unlike broad immunosuppressive therapies, antigen-specific immunotherapies aim to restore immune tolerance through selective mechanisms such as immune deviation and expanding regulatory T cells that suppress autoantigen-reactive lymphocytes. This precision mechanism reduces systemic side effects of immunosuppression and offers long-term effect through the induction of memory. In this section we evaluated the broad antigen-specific effect of loaded PS-liposomes to better understand their mechanism of action by assessing their effect on T cell repertoire and other regulatory subsets.

1. PS-liposomes exhibit appropriate characteristics and 2.5mi peptide encapsulation

PS-liposomes were generated with a specific molar ratio of DOPS/DMPC/CH to mimic an apoptotic body by presenting the PS in their surface. After the production, the PS-liposomes were characterised in terms of lipid concentration, diameter size, Pdl, Z-potential and encapsulation efficiency of the correspondent peptide (**Table 12**). To guarantee efficient phagocytosis, the PS-liposomes were consistently manufactured with a minimum diameter of 500 nm, and their concentration was maintained at 30 mM although the exact lipid concentration is not provided due to a non-standard data compilation. The negative Z-potential was determined, ensuring their potential tolerogenic properties.

Table 12. Physicochemical features of PS-liposomes used in SECTION II

Liposome nomenclature	Lipid concentration (mM)	Diameter size (nm)	Pdl	Z-potential (mV)	Encapsulation efficiency (%)
PS-empty-liposomes	~ 30	1188	0.255	-25	NA
PS-2.5mi-liposomes	~ 30	1191	0.139	-27.1	25.93

NA: not applicable; Pdl: polydispersity index.

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

To gain a deeper understanding of the mechanism of action, we aimed to address several questions related with the therapeutic process, the specific biochemical interactions and its tolerogenic effect. Although the antigen-specific expansion of Treg cells in response to PS-liposome-based therapy has been previously observed in NOD mice using tetramers, this phenomenon has not yet been the subject of a comprehensive study to understand the full antigen-specific effect on the entire T cell repertoire [388,389]. To that end, NOD.BDC2.5 mice, expressing the TCR transgene against the diabetogenic clone named BDC.2.5mi were subjected to treatment with either empty PS-liposomes, serving as a control, or PS-2.5mi-liposomes —encapsulating the 2.5mi peptide (N-start-AHHPIWARMDA-C-end), administered via IV or ID routes [392,395]. The transgenic TCR expression on CD4⁺ T cells and the effect of PS-2.5mi-liposomes on immune cell subsets was determined in the spleen at 4- or 13-days post-treatment by the splenocyte isolation, single-cell suspension and cytometry analysis (**Supplementary Figure 15**).

The viability of the splenocytes was always higher than 82% (**Figure 15A**), discarding any toxicity due to the treatment, either with empty or loaded PS-liposomes. Notably, the total number of splenocytes in NOD.BDC2.5 mice was significantly higher in those treated with 2.5mi-loaded PS-liposomes compared to mice administered with empty PS-liposomes, particularly on the fourth day following IV administration ($20.80 \pm 8.99 \times 10^6$ cells vs. $6.11 \pm 0.45 \times 10^6$ cells). Moreover, this difference was statistically significant after ID administration ($13.83 \pm 2.73 \times 10^6$ cells vs. $7.32 \pm 2.76 \times 10^6$ cells) ($p < 0.05$).

Regarding T regulatory cells, percentages of antigen-specific CD4⁺Foxp3⁺ T cells showed no differences when comparing treated and control group (**Supplementary Figure 16**). Nevertheless, after analysing Foxp3⁺ T cells subsets variations, it was found that CD4⁺CTLA4⁺Foxp3⁺ T cell subset presented a statistically significant increase in percentage and total counts after IV PS-2.5mi-liposomes treatment compared to controls at day 4 post-injection ($p < 0.05$) (**Figure 15B**). This increase was maintained until day 13 after administration when total counts were assessed. In contrast, in the ID route, both the percentage and number statistically decreased on day 13 after treatment ($p < 0.05$). Concerning CD8⁺CTLA4⁺Foxp3⁺ T cells, a significant increase in cell counts was observed in both routes four days after administration of PS-2.5mi-liposomes compared to PS-liposomes ($p < 0.05$). Following the same trend as the CD4⁺ subset, after 13 days percentage and total counts significantly decreased

to basal levels ($p < 0.05$). The non-conventional Tr1 subset, characterised by the expression of the surface markers LAG3 and CD49b, exhibited low percentages across all conditions as expected; however, interestingly total counts significantly increased four days after IV administration of PS-2.5mi-liposomes ($p < 0.05$). After 13 days of administration, percentages and total counts returned to basal levels in all conditions.

Although B cells in NODBDC2.5 mice do not express the transgenic specific 2.5mi receptor, the effect of PS-2.5mi-liposomes on B regulatory cell subsets was also studied (**Figure 15C**). Despite not reaching statistical significance, a trend towards a higher percentage and cell counts of the CD19⁺Foxp3⁺ subset was observed four days after ID administration of PS-2.5mi-liposomes, compared to mice treated with PS-empty-liposomes. Regarding the IV administration, this trend was only observed in cell counts and remarkably persisted on day 13 post-injection. Additionally, a significant increase was found in the counts of the CD19⁺CD1d⁺LAG3⁺ B cell subset four days after ID PS2.5mi-liposomes treatment, when compared to PS-empty-liposomes-treated mice ($p < 0.05$). A similar trend in percentages and total counts was noted following IV administration, although the difference did not reach statistical significance. By day 13, the increase was not sustained, and the levels of both groups returned to those of the PS-empty-liposomes group.

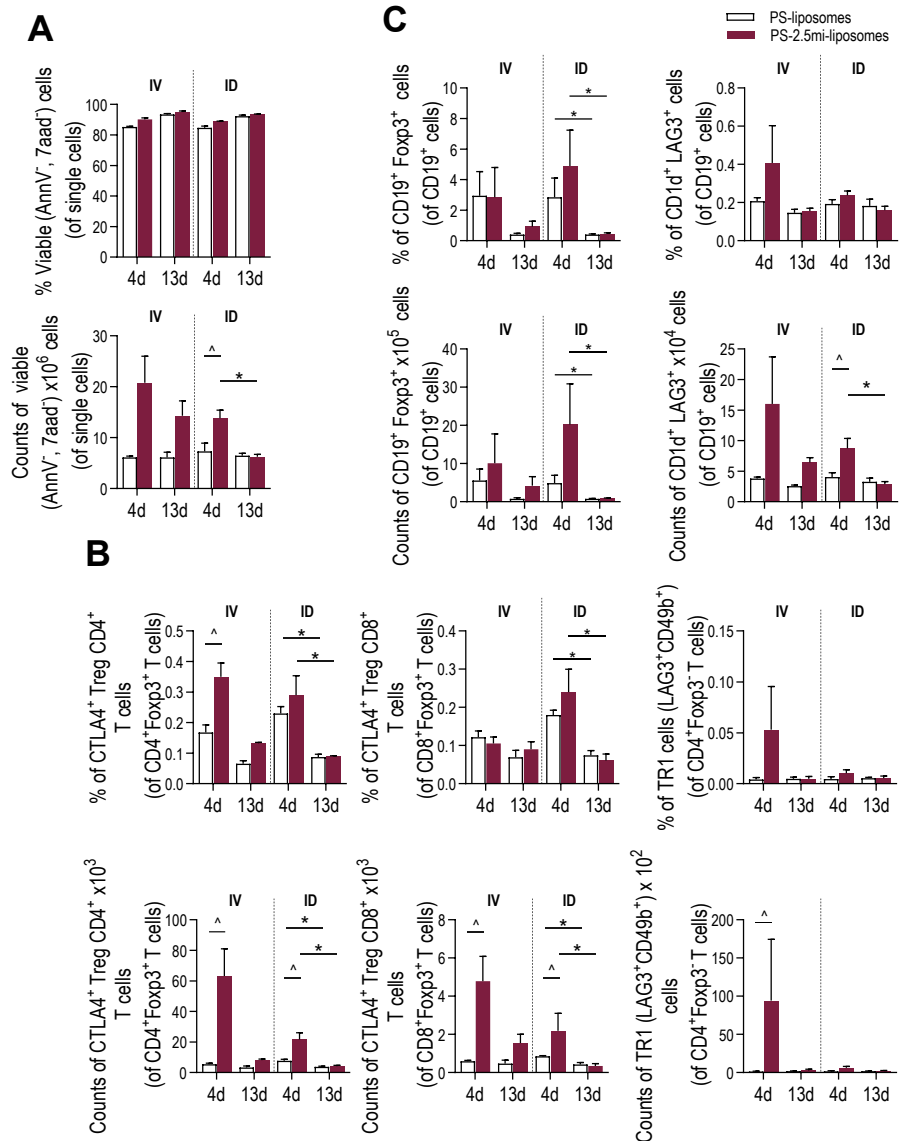


Figure 15. Percentages and cell count of regulatory T and B lymphocytes from the spleen of NOD.BDC2.5 mice after 4 or 13 days of the IV or ID PS-liposomes or PS2.5mi-liposomes administration, measured by flow cytometry (n=4). **(A)** Percentage and total counts of viable cells, **(B)** Treg cell subsets –CD4⁺CTLA4⁺Foxp3⁺, CD8⁺CTLA4⁺Foxp3⁺ and Tr1 CD4⁺Foxp3⁺LAG3⁺CD49b⁺ T cells, **(C)** Breg-cell subsets –CD19⁺Foxp3⁺ and CD19⁺CD1d⁺LAG3⁺ B cells–. Bars represent PS-empty-liposomes (white) and PS-2.5mi-liposomes (dark pink). Data represented as mean ± SEM. Significant differences were found when comparing when comparing the groups (^p < 0.05 between liposome treatment, *p < 0.05 between timepoints, Mann-Whitney test). From Almenara-Fuentes et al., 2023.

Anergic and exhausted T cells, characterised by their diminished effector functions, can play a pivotal role in establishing long-term tolerance by prevention of the proliferation of autoreactive T cells and contributing to suppress autoimmune responses [403]. Here we explored the potential of those subsets after the PS-liposomes administration, regarding the antigen-specific anergic CD4⁺ T cell compartment, it was found that CD73⁺FR4⁺ subset 4 days after treatment, no increase was detected (**Figure 16A**). However, PS-2.5mi-liposomes administered intravenously after 13 days presented an increase in percentage and total counts, although non-significance. Similarly, anergic CD8⁺ T cells showed a trend to increase their counts after 4 and 13 days of the IV PS-2.5mi-liposomes injection (**Figure 16B**). Antigen-specific exhausted CD4⁺ T cells presented statistically significant higher counts 4 days after PS-2.5-mi-liposomes ID administration in comparison to control group ($p < 0.05$), although non significance, IV administration of loaded PS-liposomes also resulted in a total count increase (**Figure 16C**).

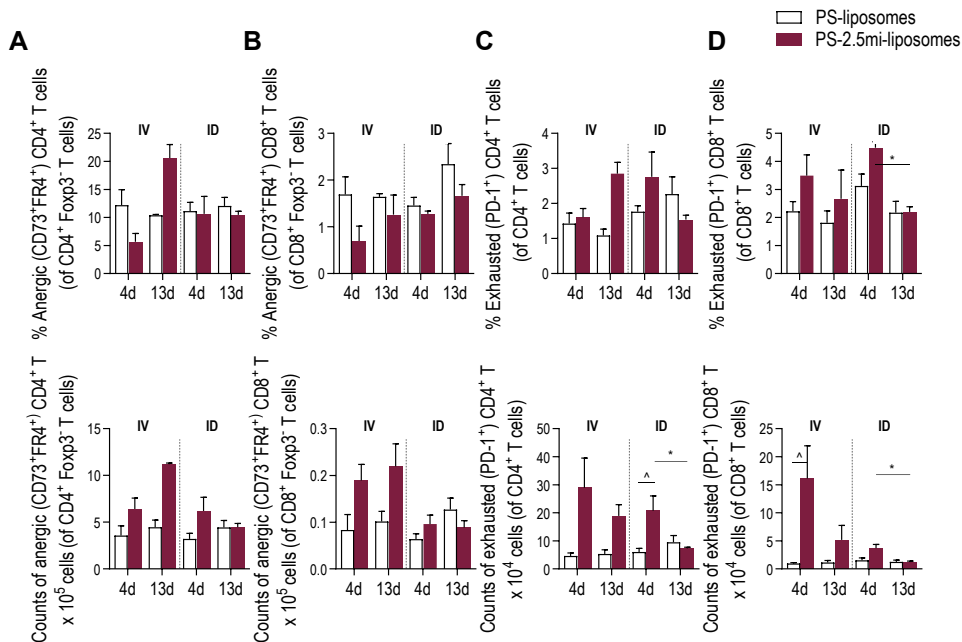


Figure 16. Percentages and cell count of anergic and exhausted T cells from the spleen of NOD.BDC2.5 mice after 4 or 13 days of the IV or ID PS-liposomes or PS2.5mi-liposomes administration (n=4). (A) Percentages and total cell count of anergic CD4⁺ T cells – CD4⁺Foxp3⁺CD73⁺FR4⁺ T cells–, (B) anergic CD8⁺ T cells – CD8⁺Foxp3⁺CD73⁺FR4⁺ T cells–, (C) exhausted CD4⁺ T cells – CD4⁺PD-1⁺ T cells–, (D) exhausted CD8⁺ T cells – CD8⁺PD-1⁺ T cells–. Bars represent PS-empty-liposomes (white) and PS-2.5mi-liposomes (dark pink). Data represented as mean $\pm$ SEM. Significant differences were found when comparing when comparing the groups (^p < 0.05 between liposome treatment, *p < 0.05 between timepoints, Mann-Whitney test). From Almenara-Fuentes et al., 2023.

Remarkably, after IV administration of PS-2.5mi-liposomes the percentage and total counts of exhausted CD4⁺ T cells remained elevated even 13 days post-treatment. Furthermore, a significant increase in exhausted CD8⁺ T cell counts was also observed 4 days after IV treatment ($p<0.05$) and a tendency after ID administration (**Figure 16D**). The sustained higher counts of T exhausted cells were also seen in the CD8⁺ compartment after IV administration.

Regarding other T cell subsets –Naïve, Effector, Central Memory and Effector Memory T cells subsets, a notable shift was observed following PS-2.5mi-liposome administration. 13 days after IV treatment, the frequency of Naïve CD4⁺ T cells significantly declined, accompanied by a corresponding increase in the Effector Memory CD4⁺ T cell population, when compared to mice receiving PS-empty-liposomes (**Figure 17A**). On the other hand, in the CD8⁺ T cell compartment, this shift occurred earlier, being evident as soon as day 4 post-treatment. At this time point, a significant reduction in Naïve CD8⁺ T cells and a concomitant increase in Effector Memory CD8⁺ T cells were detected relative to the PS-empty-liposome group (**Figure 17B**). ID administration followed a similar trend; however, these changes did not reach statistical significance. Importantly, the relative frequencies of Central Memory and Effector T cell subsets remained unchanged, regardless of treatment group, route of administration, or time point analysed.

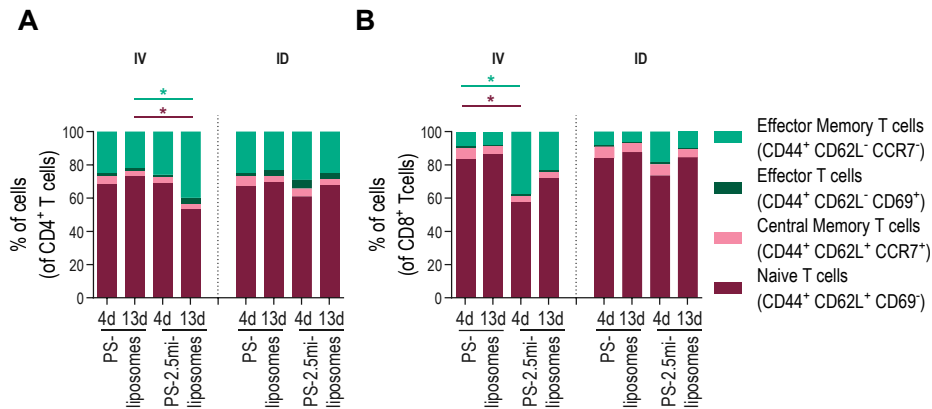


Figure 17. Percentages of Naïve, Effector, Central Memory and Effector Memory T cells from the spleen of NOD.BDC2.5 mice after 4 or 13 days of the IV or ID PS-liposomes or PS2.5mi-liposomes administration (n=4). (A) CD4⁺ T cell compartment, (B) CD8⁺ T cell subsets. Bars represent the relative percentage of Naïve T cells (dark pink), Central Memory T cells (light pink), Effector T cells (dark green) and Effector Memory T cells (light green). Data represented as mean. Significant differences were found when comparing the different subsets among groups (*p < 0.05, ordinary one-way ANOVA with Tukey's multiple comparisons test).

SECTION III. PS-liposome effect on human immune cells

The third part of this work aimed at determining the tolerogenic effects of PS-liposomes on human immune cells from MG and RA patients. To that end, we isolated monocytes from peripheral blood mononuclear cells or synovial fluid, and differentiated them into DCs. Following treatment with antigen-specific loaded PS-liposomes, the effects on DCs phenotype were assessed to better understand the tolerogenic potential. Subsequently, the DCs were cocultured with the autologous PBMCs to examine the tolerogenic peptide presentation and evaluate their tolerogenic functionality. To explore the effect of PS-loaded-liposomes on other immune cells, cocultures were also carried out with PBMCs from healthy donors or patients with MG and RA.

1. PS-liposomes efficiently encapsulate human MG and RA autoantigen peptides

For these experiments, PS-liposomes were generated with a specific molar ratio of DOPS/DMPC/CH to emulate an apoptotic body presenting the PS in their surface. To produce the fluorescent PS-liposomes NBD staining was introduced in the process of PS-liposome elaboration. A summary of the physicochemical features is found in **Table 13**. To guarantee phagocytosis, the PS-liposomes were always intended to have a minimum diameter of 500 nm, and a concentration around 30 mM. The ζ -potential was always negative, ensuring their tolerogenic potential. Variations in encapsulation efficiency among different peptides were observed, which can be attributed to the peptides' charges, with negatively charged peptides being more effectively encapsulated by the negatively charged PS-liposomes than the positive ones.

Table 13. Physicochemical features of PS-liposomes used in SECTION III

Liposome nomenclature	Number of batches	Lipid concentration (mM)	Diameter size (nm)	PdI	Z-potential (mV)	Encapsulation efficiency (%)
NBD-PS-AChR-liposomes	4	29.9 ± 1.22	521 ± 94.01	0.29 ± 0.07	-22.95 ± 1.42	74.72 ± 37.00
NBD-PS-MCV-liposomes	4	28.86 ± 2.99	891.65 ± 135.06	0.30 ± 0.01	-22.25 ± 3.28	90.09 ± 8.55
NBD-PS-empty-liposomes	3	26.16 ± 3.15	776.57 ± 79.50	0.37 ± 0.057	-23.57 ± 3.18	NA
PS-AChR-liposomes	5	27.32 ± 2.99	772.10 ± 373.97	0.28 ± 0.06	-22.23 ± 1.47	78.88 ± 20.66
PS-MCV-liposomes	5	27.55 ± 1.52	852.16 ± 238.62	0.30 ± 0.12	-22.66 ± 3.02	87.43 ± 13.92
PS-empty-liposomes	5	28.23 ± 2.42	838.32 ± 406.61	0.30 ± 0.11	-21.34 ± 1.23	NA
PS-SPACER-liposomes	1	25.6	1054 ± 52	0.25 ± 0.01	-28.50 ± 1.10	44.96

Data presented as mean ± SD; AChR: acetyl choline receptor; MCV: mutated citrullinated vimentin; NA: not applicable; NBD; nitrobenzoxadiazole; PdI: polydispersity index.

2. PS-loaded-liposomes are effectively stained with the pHrodo green dye

Aiming to assess if a specific immune cell subset was able to phagocytose PS-liposomes, a novel post-production method to fluorescently label the PS-liposomes was developed using the pH-sensitive pHrodo green dye, which exhibits increased fluorescence in acidic environments. Given that the polar head of PS in the liposomes contains an amino group, the pHrodo amino-reactive dye was incubated with the liposomes to facilitate the staining process. Following a thorough dialysis procedure to remove unbound dye, the fluorescently stained PS-liposomes were tested. As expected, results demonstrated a progressive increase in FITC fluorescence intensity as the pH of the media decreased (**Figure 18**).

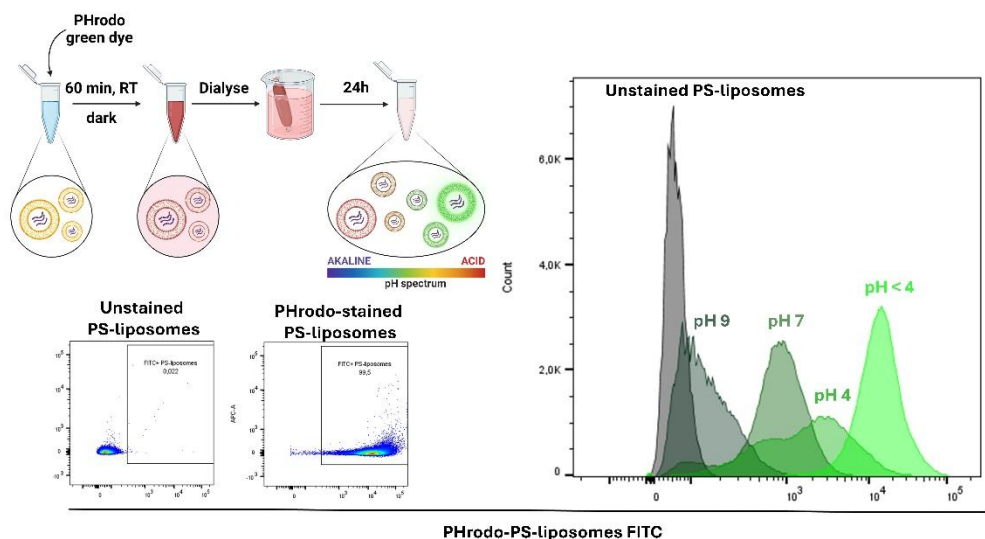


Figure 18. pHrodo-stained PS-AChR-liposomes emit FITC fluorescence only in acid media. Protocol of pHrodo staining PS-liposomes and histogram of the FITC fluorescence of the pHrodo stained PS-liposomes after the incubation in solutions with different pH values.

3. Comparison of gender, age, and BMI characteristics between adult control subjects and patients with MG and RA

To investigate the phenotypic and functional changes in DCs induced by phagocytosis of PS-loaded-liposomes, 11 patients with MG from Hospital de Sant Pau and 12 patients with RA from Hospital Vall d'Hebron were recruited, each donating 50 ml of blood. To investigate the phenotypic and functional changes in DCs induced by phagocytosis of PS-loaded-liposomes, 11 patients with MG from Hospital de Sant Pau (Barcelona, Spain) and 12 patients with RA from Hospital Vall d'Hebron (Barcelona, Spain) were recruited, each donating 50 ml of peripheral blood. For control purposes, 19 healthy subjects were recruited. All participants met the inclusion criteria and provided informed consent for study. The most relevant clinical data of the subjects is summarised in **Table 14**. All MG patients were positive for anti-AChR autoantibodies and most of them were classified as a generalized form of MG, only one patient presented thymic hyperplasia (**Supplementary Table 1**). The age of onset of the disease is in accordance with the literature, with women presenting an early incidence while late onset patients are predominantly men. [92]. 9 of the 12 RA patients presented positivity for ACPA and were carriers of the DRB1*04 haplotype (**Supplementary Table 2**). Moreover, presented a DAS28 score always higher than 3.2, indicating a moderate-to-high disease activity.

Table 14. Data from the patients and control subjects included in this study

Parameter	MG patients	RA patients	Control subjects	p-value
n	11	12	19	-
Sex	2/11 (18%) Female 9/11 (82%) Male	11/12 (92%) Female 1/12 (8%) Male	12/19 (63%) Female 7/19 (37%) Male	-
Age (years)	66.73 ± 10.36	63.17 ± 18.63	29.89 ± 5.90	<0.0001 <0.0001
Age at diagnosis	64.27 ± 14.25	48.92 ± 21.46	NA	0.066
Years of progression	2.45 ± 4.78	14.25 ± 14.69	NA	<0.01
Anti-AChR (nmol/L)	61.64 ± 47.74	NA	NA	-
DAS28	NA	4.69 ± 1.19	NA	-

Data presented as mean ± SD; p-value calculated from Mann-Whitney test. RA: rheumatoid arthritis; BMI: body mass index; MG; Myasthenia Gravis; NA: not applicable.

4. Monocytes from MG and RA patients efficiently differentiate to DCs and efficiently uptake PS-liposomes

Monocytes were isolated from PBMCs to be differentiated into DCs. The yield of monocyte isolation—calculated from the absolute number of CD14⁺ cells obtained in the positive isolated fraction related to the absolute number of CD14⁺ cells in total PBMCs—, the purity of CD14⁺ cells, the viability and the differentiation efficiency of the samples is shown in **Table 15**. The viability of DCs, both immature and mature, cultured with or without PS-empty liposomes or PS-loaded liposomes, was evaluated to determine the potential toxicity of the PS-liposomes (data not shown). DCs from MG and from RA patients consistently showed a viability higher than 90% across all conditions, confirming again that PS-liposomes at a concentration of 1mM maintain viability.

Table 15. Data on the isolation of monocytes and their differentiation into DCs from adult subjects with MG and RA.

Parameter	MG patients	AR patients	p-value
Yield at day 1 (%)	94.96 ± 29.49	80.37 ± 14.23	0.654
CD14 purity at day 1 (%)	79.56 ± 4.88	81.45 ± 5.92	0.342
Viability at day 1 (%)	97.17 ± 3.74	97.74 ± 1.30	0.487
Differentiation efficiency, at day 6 (CD11c ⁺ %)	91.38 ± 4.70	92.01 ± 5.97	0.556

Data presented as mean ± SD; p-value calculated from Mann-Whitney test. Yield: The percentage of the absolute number of CD14⁺ cells in the positively isolated fraction relative to the absolute number of CD14⁺ cells in the total PBMCs. CD14 purity: the percentage of CD14⁺ cells in the isolated fraction. Viability: percentage of 7-AAD and Annexin V negative cells of isolated CD14⁺ cells. Differentiation efficiency: percentage of CD11c⁺ cells.

5. IDCs exhibit higher phagocytic ability than mDCs

Human monocyte-derived DCs have previously demonstrated their ability to efficiently phagocytose PS-liposomes [390]. However, in this study, the phagocytic capacity of DCs from patients with MG and RA was tested for the first time. To assess this capacity, iDCs and mDCs were cocultured with NBD-PS-loaded-liposomes for 24h with the aim of detecting those cells positive for the NBD fluorescence (**Supplementary Figure 17**). As shown in **Figure 19**, both iDCs and mDCs conditions showed a high percentage of positivity for the fluorescence emitted by the NBD-labelled PS-loaded-liposomes, regardless the disease. These results were consistently and significantly different when compared to their respective control DCs conditions ($p < 0.0001$). Nevertheless, in MG patients was observed that iDCs ($85.10 \pm 11.00\%$) had a significant higher enhanced phagocytic capacity for PS-AChR-liposomes compared to mDCs ($66.93 \pm 9.30\%$) ($p < 0.05$). In RA patients, this difference was also statistically significant ($p < 0.01$), with NBD positivity levels of $91.57 \pm 6.15\%$ in iDCs compared to $77.30 \pm 4.35\%$ in mDCs.

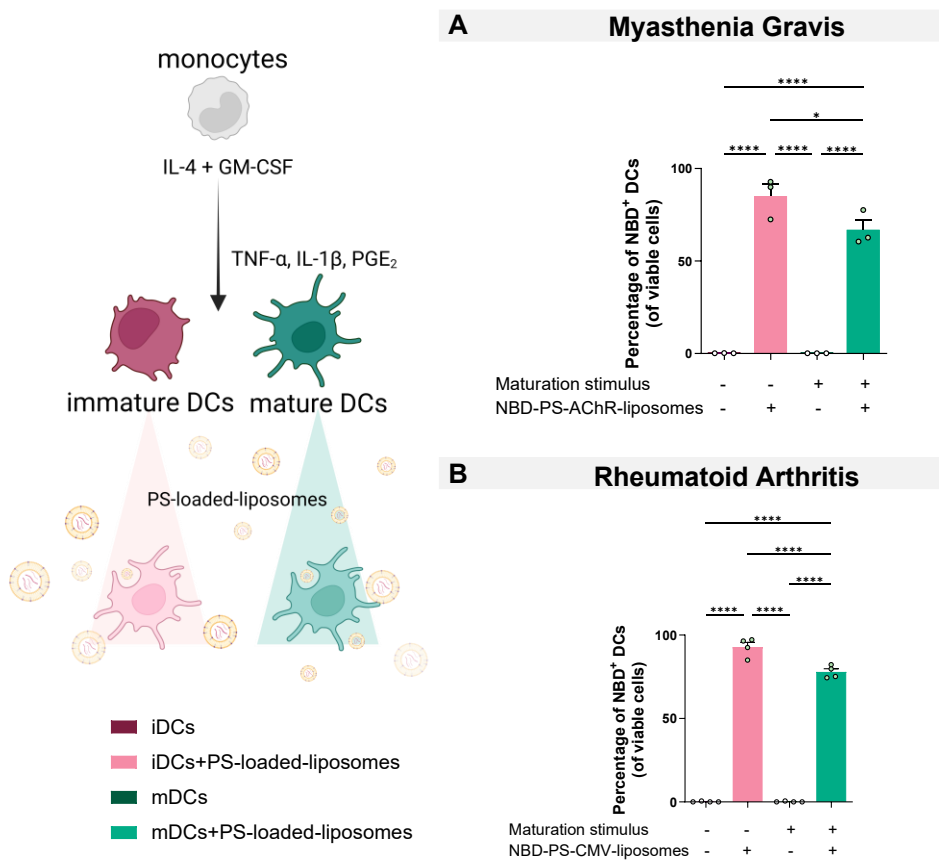


Figure 19. Percentage of NBD⁺ DCs from MG and RA patients after NBD-PS-loaded-liposomes coculture. Scheme of the protocol of monocyte derived DCs with the final 4 different evaluated conditions (left panel), **(A)** DCs obtained from MG ($n \geq 3$) and **(B)** RA patients ($n \geq 3$). Bars represent iDCs (dark pink), iDCs after PS-loaded-liposome phagocytosis (light pink), mDCs (dark green) or mDCs after PS-loaded-liposome phagocytosis (light green). Data represented as mean $\pm$ SEM. Significant differences were found when comparing among culture conditions (**** $p < 0.0001$, ** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test).

6. PS-liposomes efferocytosis modulates phenotype in both iDCs and mDCs from MG and RA patients

After confirming that monocyte-derived DCs from MG and RA patients were able to engulf PS-liposomes, the next step was to assess the phenotypic changes that might

result from this efferocytosis process. Initially, the expression of PS receptors, including TIM4, CD36, and integrin $\alpha\text{v}\beta 5$, was assessed to determine whether their expression was modulated by the PS stimulus from the liposomes. As expected, iDCs from both MG and RA patients showed expression of all studied PS-receptors (**Figure 20**). Furthermore, in comparison to iDCs, the mDCs condition was observed to present lower levels of TIM-4 expression ($p < 0.01$) and CD36 expression ($p < 0.001$ in MG patients and $p < 0.01$ in RA patients), whereas integrin $\alpha\text{v}\beta 5$ only showed a tendency. After the PS-loaded-liposomes coculture, immature conditions from MG patients presented a tendency to increase the CD36 expression in comparison to iDCs condition, although not significant. Nevertheless, RA patients did present a statistically significant increase in the three PS receptors when iDCs engulfed PS-MCV-liposomes ($p < 0.01$ and $p \leq 0.05$). When iDCs+PS-loaded-liposomes condition was compared to mDCs control condition a significant upregulation was always present, in both MG and RA patients ($p < 0.001$, $p < 0.01$, and $p \leq 0.05$), however, integrin $\alpha\text{v}\beta 5$ in MG patients only showed a tendency. In comparison to mDCs that have engulfed PS-loaded-liposomes, it was always observed a statistically significant increase of TIM4 and CD36 expression in both MG and RA patients ($p < 0.001$ and $p < 0.01$). Regarding the mDCs+PS-loaded-liposomes condition, despite observing a tendency to increase in comparison to the respective mDCs control condition, it was only significantly increased in RA patients at TIM-4 receptor. When compared this condition with iDCs condition, the expression of CD36 was statistically reduced in both MG and RA patients ($p < 0.01$ and $p \leq 0.05$) while TIM4 expression only in MG patients ($p < 0.01$). iDCs and mDC conditions cultured with empty PS-liposomes, in both MG and RA conditions, followed the same trends as the PS-loaded-liposomes (**Supplementary Figure 18**).

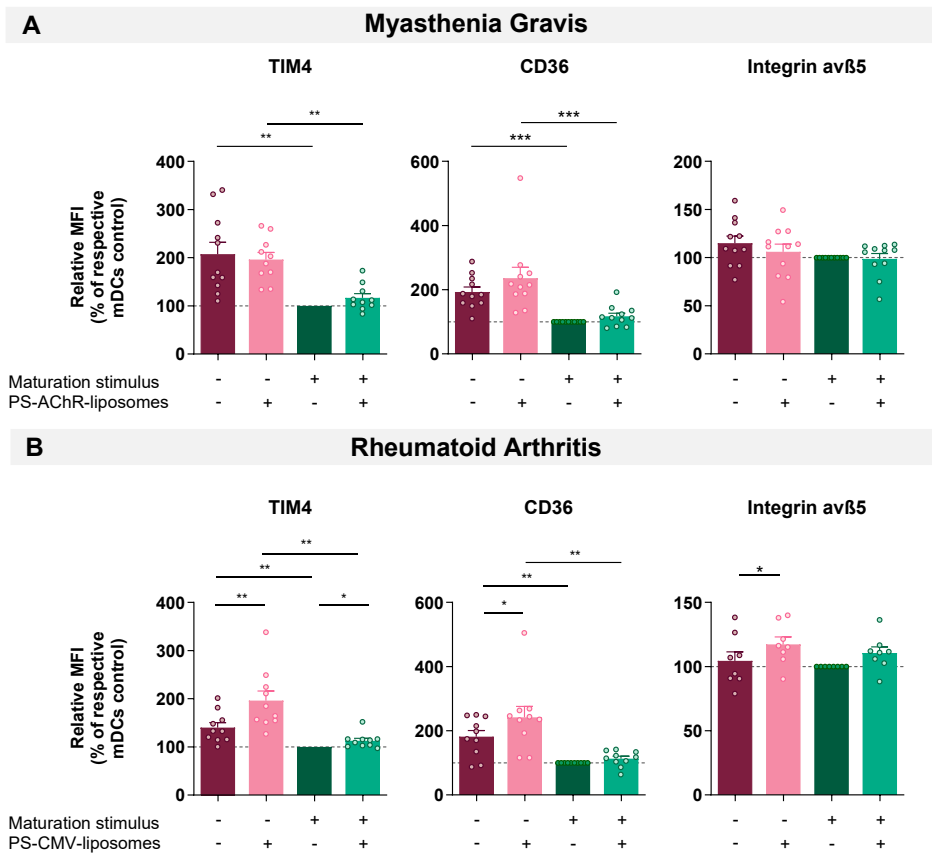


Figure 20. PS receptors are modulated in DCs after PS-loaded-liposomes phagocytosis. (A) Relative MFI values of TIM-4, CD36 and Integrin $\alpha\beta 5$ membrane expression in iDCs and mDCs obtained from MG ($n \geq 11$) and **(B)** RA patients ($n \geq 12$). Bars represent iDCs (dark pink), iDCs after PS-loaded-liposome phagocytosis (light pink), mDCs (dark green) or mDCs after PS-loaded-liposome phagocytosis (light green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions (** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition (** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, One sample Wilcoxon signed-rank test).

Afterwards, the expression of the selected immune phenotype markers was evaluated to study their modulation after DCs phagocyte the PS-loaded-liposomes (**Figure 21 A and B**). Regarding the antigen-presenting molecules, HLA-DR marker showed, as expected, a statistically significant lower expression in iDCs conditions when compared to mDCs control conditions, in both MG and RA patients ($p < 0.01$). Conditions cocultured with PS-loaded liposomes showed a tendency to increase the

expression of this marker, in iDCs after the engulfment it was found to be statistically significant higher compared to the respective iDCs condition in RA patients ($p \leq 0.05$). Nevertheless, HLA-DR expression values in iDCs PS-loaded-liposomes conditions were lower in comparison to mDCs with PS-loaded-liposomes, showed only a tendency in RA patients but being statistically significant in MG patients ($p < 0.01$). Furthermore, although no significance, mDCs after PS-loaded-liposomes phagocytosis showed an increase in HLA-DR expression in comparison to control mDCs. HLA-ABC expression was similar in all DCs conditions, showing no statistical differences in MG patients. Nevertheless, DCs from RA patients tended to increase the expression of this marker after the PS-MCV-liposomes uptake, being statistically significant when compared between iDCs and iDCs with PS-MCV-liposomes ($p \leq 0.05$) but not significant when compared mDCs conditions.

The expression of costimulatory molecules such as CD40 and CD86 was always lower in iDCs than mDCs control condition in both groups of patients ($p < 0.0001$, $p < 0.001$ and $p \leq 0.05$). PS-loaded-liposomes engulfment did not increase the expression levels of these two markers in iDCs conditions, being also statistically lower in comparison to mDCs control condition ($p < 0.0001$ and $p < 0.01$), with the exception of the CD40 marker in RA patients which only showed a trend. When compared to mDCs with PS-loaded-liposomes, iDCs engulfing PS-loaded liposomes also showed a statistically significant reduction in both markers and both patient's groups ($p < 0.01$ and $p \leq 0.05$), except for CD40 marker in RA patients. However, mDCs after the uptake of the PS-loaded-liposomes showed a different trend on CD40 and CD86. While CD40 expression tended to increase in both MG and RA patients in comparison to mDCs control condition, in CD86 expression tended to decrease.

The expression of the CD54 adhesion molecule was statistically lower in iDCs in comparison to mDCs control condition in MG patients ($p < 0.0001$), and RA patients displayed the same trend. When compared to iDCs cocultured with PS-loaded-liposomes, CD54 expression showed a downregulation, being statistically significant in RA patients ($p \leq 0.05$). Moreover, this downregulation displayed to be statistically significant when compared with mDCs engulfing PS-loaded-liposomes in both MG and RA patients ($p < 0.01$). Downregulation following uptake of PS-loaded liposomes was also observed in the mDCs conditions, as mDCs from MG patients had statistically lower CD54 expression compared to the control mDCs condition ($p \leq 0.05$), a trend that was also found in mDCs after the PS-MCV-liposome uptake, although non significance. CD25 expression was found to be lower in iDCs than control mDCs conditions in both MG and RA patients ($p < 0.001$ and $p \leq 0.05$). PS-loaded-liposome

uptake did not modulate CD25 expression, neither in immature nor mature conditions in comparison to their respective DCs condition.

In this way, after the PS-loaded-liposome engulfment iDCs maintained statistically significant low levels of expression in comparison to control mDCs conditions ($p < 0.001$ and $p < 0.01$) and to mDCs cultured with PS-loaded-liposomes ($p < 0.01$). PS-empty-liposomes, in general, presented the same trends in all conditions except for the decrease in CD54 and CD86 markers on matured DCs conditions (**Supplementary Figure 19**).

Furthermore, the expression of CCR7 and CXCR4 chemokine receptors, CD14 and DC-SIGN pattern recognition receptors and the PD-L1 immune checkpoint inhibitor was assessed in the different DCs conditions (**Figure 22 A and B**). Chemokine receptor CCR7 did not show any statistical differences among groups in any of the patients with the diseases studied. Nevertheless, CXCR4 expression, as expected, presented a statistically lower expression in iDCs conditions when compared to mDCs control conditions, in both MG and RA patients ($p < 0.00001$). Remarkably this marker presented an overexpression in conditions cocultured with PS-loaded-liposomes. iDCs from MG patients were significantly increased after coculture with PS-AChR liposomes ($p \leq 0.05$), while RA iDCs only exhibited the trend with PS-MCV liposomes. iDCs cocultured with PS-loaded-liposomes remained statistically lower compared, to mDCs cocultured with the corresponding PS-loaded-liposomes ($p < 0.0001$ and $p < 0.001$) in both MG and RA patients, respectively. Interestingly, mature conditions with PS-loaded-liposomes showed a consistent statistically significant overexpression of the CXCR4 marker in comparison to the control mDCs condition ($p < 0.0001$ and $p < 0.001$).

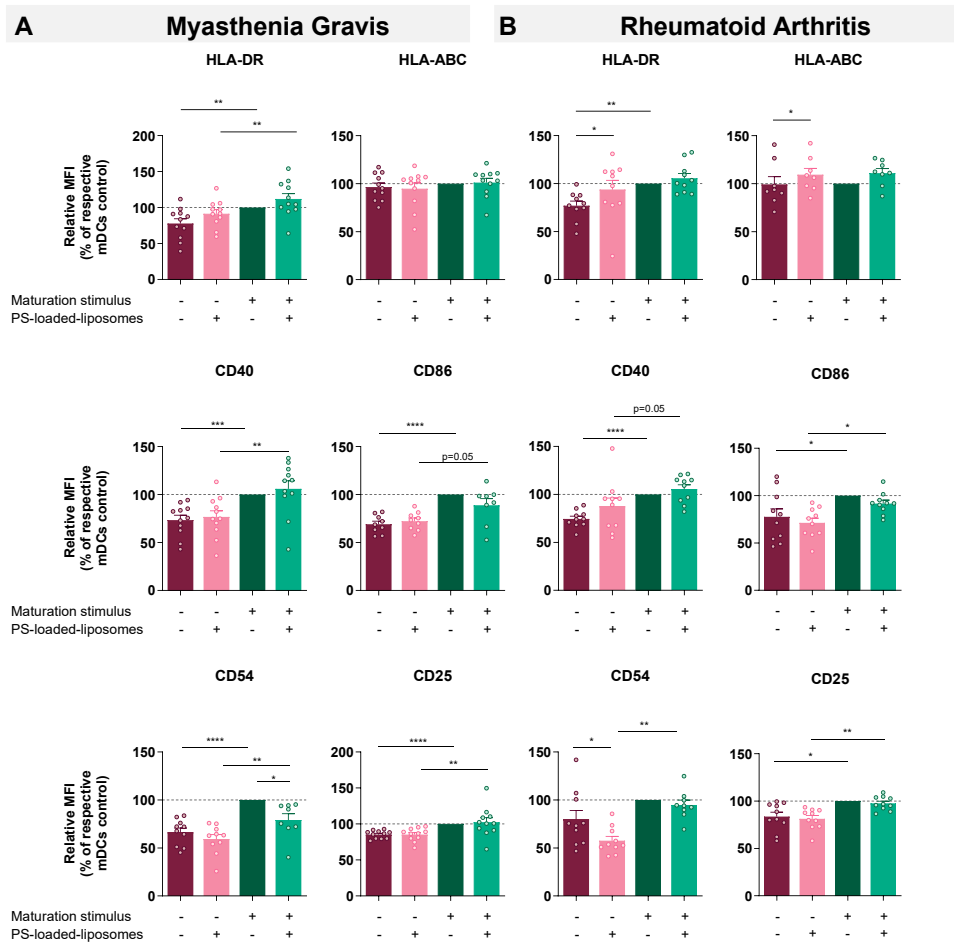


Figure 21. PS-loaded-liposomes phagocytosis modulates immune phenotype markers in DCs. (A) Relative MFI values of HLA-DR, HLA-ABC, CD40, CD86, CD54 and CD25 membrane expression in iDCs and mDCs obtained from MG ($n \geq 11$) and **(B)** RA patients ($n \geq 12$). Bars represent iDCs (dark pink), iDCs after PS-loaded-liposome phagocytosis (light pink), mDCs (dark green) or mDCs after PS-loaded-liposome phagocytosis (light green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions (*** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, One sample Wilcoxon signed-rank test).

The CD14 pattern recognition marker presented a statistical downregulation in iDCs conditions compared to control mDCs condition ($p \leq 0.05$) in both MG and RA patients. Moreover, when iDCs were cocultured with PS-loaded-liposomes, the expression of this marker resulted in a statistical increase in comparison to the respective iDCs condition ($p \leq 0.05$). MG patients showed an upregulation after PS-AChR-liposomes coculture in mDCs, compared to iDCs with PS-AChR-liposomes ($p \leq 0.05$).

Regarding DC-SIGN, there was found a tendency to increase in iDCs condition after the PS-loaded-administration, being statistically increased compared to mDCs with PS-AChR-liposomes in MG patients ($p \leq 0.05$). Finally, PD-L1 marker presented a downregulation in iDCs compared to control mDCs conditions in both MG and RA patients ($p < 0.001$ and $p < 0.0001$) but no significant differences compared to iDCs cocultured with PS-loaded-liposomes. This downregulation remained when iDCs with PS-loaded-liposomes were compared with mDCs cocultured with PS-loaded-liposomes ($p \leq 0.05$). Interestingly, mDCs after the Ps-loaded-liposome uptake presented statistically significant higher expression levels in comparison to control mDCs in MG patients ($p \leq 0.05$), and the same trend in AR patients although not statistically significant. IDCs and mDCs conditions cultured with empty PS-liposomes, in both MG and RA conditions, followed the same trends as the PS-loaded-liposomes, except for PD-L1 which did not show the same trend to increase as mDCs cultured with PS-loaded-liposomes (**Supplementary Figure 20**).

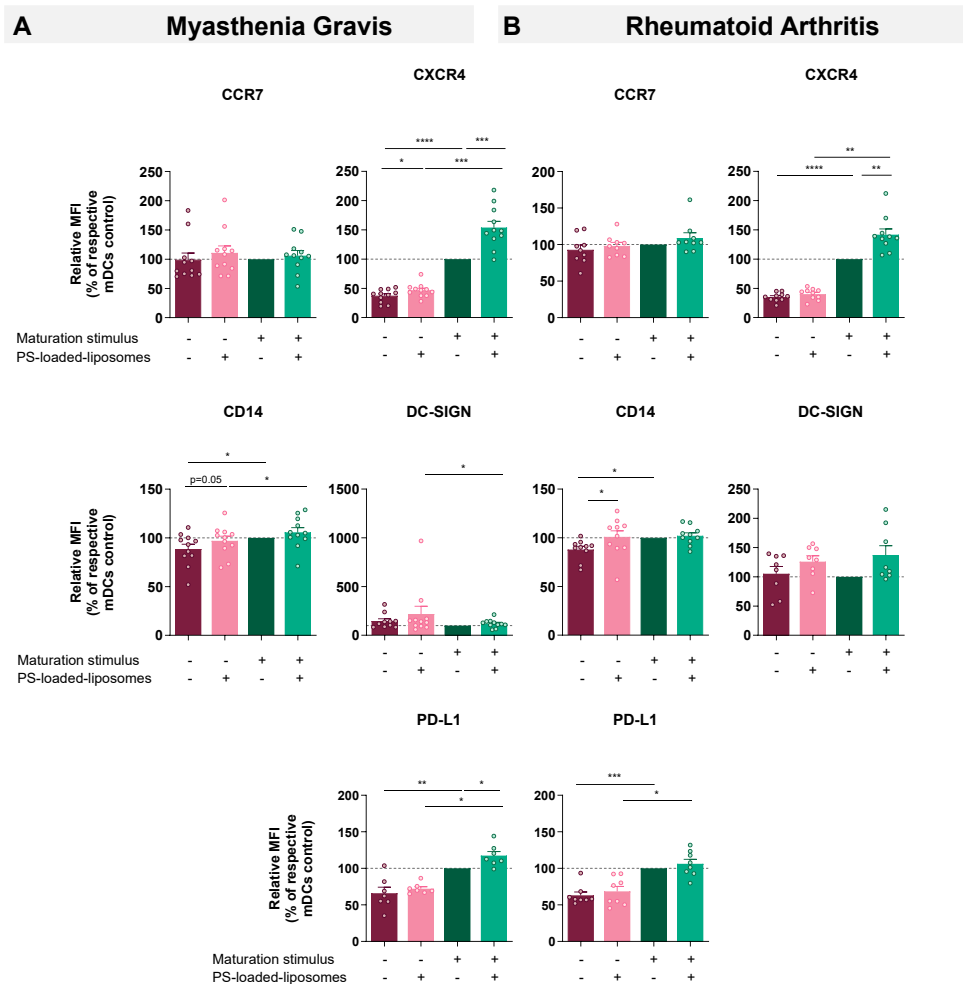


Figure 22. PS-loaded-liposomes phagocytosis modulates immune phenotype markers in DCs. (A) Relative MFI of CCR7, CXCR4, CD14, DC-SIGN and PD-L1 membrane expression in iDCs and mDCs obtained from MG ($n \geq 11$) and **(B)** RA patients ($n \geq 12$). Bars represent iDCs (dark pink), iDCs after PS-loaded-liposome phagocytosis (light pink), mDCs (dark green) or mDCs after PS-loaded-liposome phagocytosis (light green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions ($***p < 0.001$, $**p < 0.01$, $*p \leq 0.05$, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition ($***p < 0.0001$, $**p < 0.001$, $*p \leq 0.05$, One sample Wilcoxon signed-rank test).

7. NBD-labelled PS-liposomes capture confirmed the phenotype modulation effect on mature and iDCs

To explore more precisely how PS-loaded-liposomes efferocytosis affects the biology of DCs, the same conditions of previously differentiated DCs were cultured with fluorescent liposomes labelled with NBD fluorochrome. After 24h culture, flow cytometry enabled to discern between DCs that had phagocytosed, positive for the fluorescence emitted by the NBD fluorochrome, and those that had not, negative for NBD (**Supplementary Figure 17**).

Interestingly, those iDCs that phagocytosed NBD-labelled PS-loaded-liposomes tended to present higher levels of PS receptors, either TIM-4 and CD36, in both MG and RA patients, when compared to those iDCs unable to phagocytose them, but also to those iDCs in basal conditions (**Figure 23**). In the mDCs compartment, although the levels were lower than iDCs, those cells engulfing PS-loaded-liposomes displayed the same biological tendency to upregulate both PS-receptor markers, although non-significant.

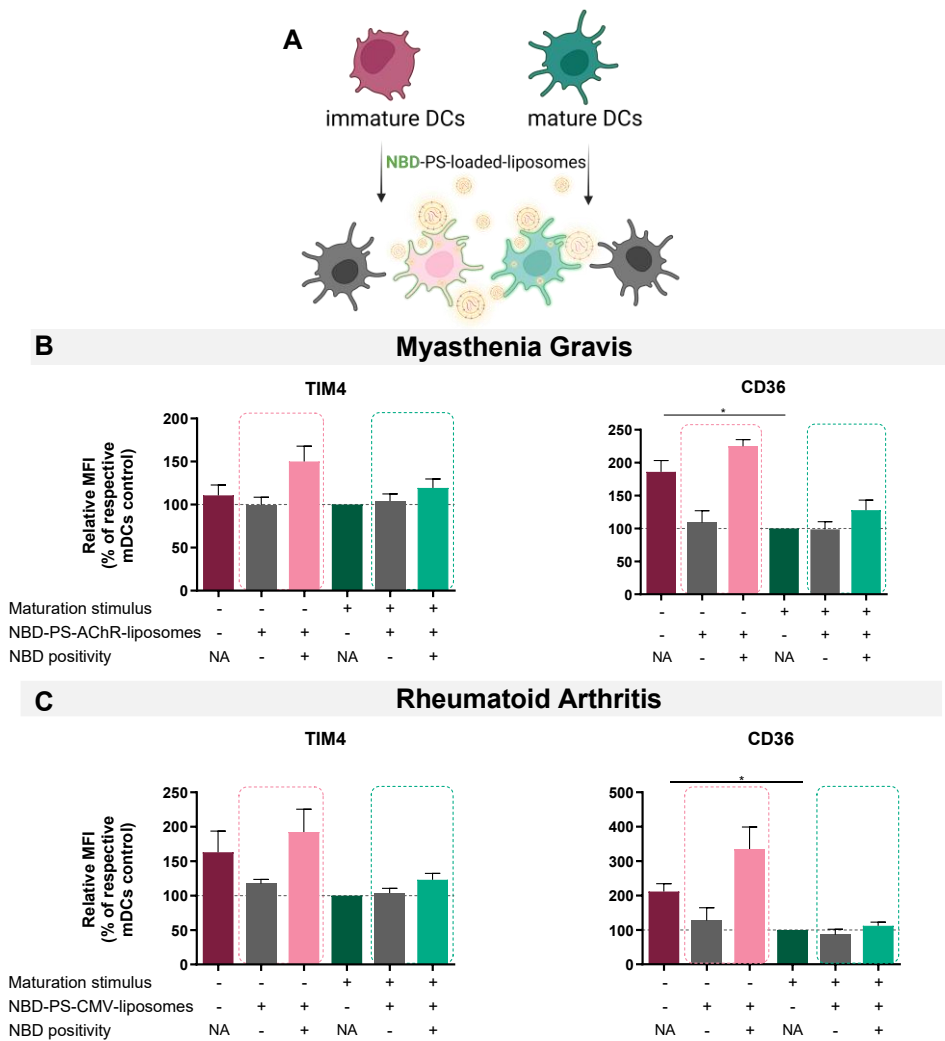


Figure 23. Membrane expression of PS receptors in DCs after NBD-PS-loaded-liposomes phagocytosis. (A) Scheme of the six different DCs evaluated. (B) Relative MFI of TIM-4 and CD36 in iDCs and mDCs obtained from MG (n=3-4) and (C) RA patients (n=3-4). Bars represent iDCs (dark pink), iDCs not positive for NBD fluorescence after NBD-PS-loaded-liposome coculture (grey), iDCs positive for NBD fluorescence after NBD-PS-loaded-liposome coculture (light pink), mDCs (dark green), mDCs not positive for NBD fluorescence after NBD-PS-loaded-liposome coculture (grey) or mDCs positive for NBD fluorescence after NBD-PS-loaded-liposome coculture (light green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing a culture condition with the mDCs control condition (**p<0.01, *p $\leq$ 0.05, One sample Wilcoxon signed-rank test).

Regarding the other surface markers (**Figure 24**), upon engulfment of PS-loaded-liposomes, iDCs exhibited CD40 surface expression levels comparable to those observed under basal immature conditions. In contrast, mDCs demonstrated a tendency toward increased CD40 expression; however, this increase was not statistically significant. Notably, CD40 expression across all iDC conditions consistently remained lower than that observed in basal mDCs. Interestingly, in alignment with prior findings using non-labelled PS-loaded liposomes, CD54 expression in mDCs tended to decrease following PS-liposome uptake when compared to basal mDC conditions. Regarding CCR7 expression, basal iDCs and non-engulfing iDCs displayed low levels of this marker. However, iDCs that internalized PS-liposomes showed a marked tendency in upregulation of CCR7, particularly in RA patient samples. This upregulation was also observed slightly in mDCs after PS-liposome uptake. Similarly, the CXCR4 marker exhibited increased expression in engulfing DCs, with a modest but discernible elevation in iDCs and a remarkably increase in mDCs compartment. Conversely, CXCR4 expression remained unchanged in basal and non-engulfing DCs across both immature and mature conditions. The anti-inflammatory PD-L1 molecule expression tended to be upregulated in DCs capable of phagocytosing PS-liposomes, specifically in RA patient samples, consistent with previous observations using non-fluorescent PS-liposomes. Lastly, DC-SIGN expression was elevated following PS-liposome uptake in both iDCs and mDCs, with the increase being more pronounced in RA DCs, particularly in the immature subset.

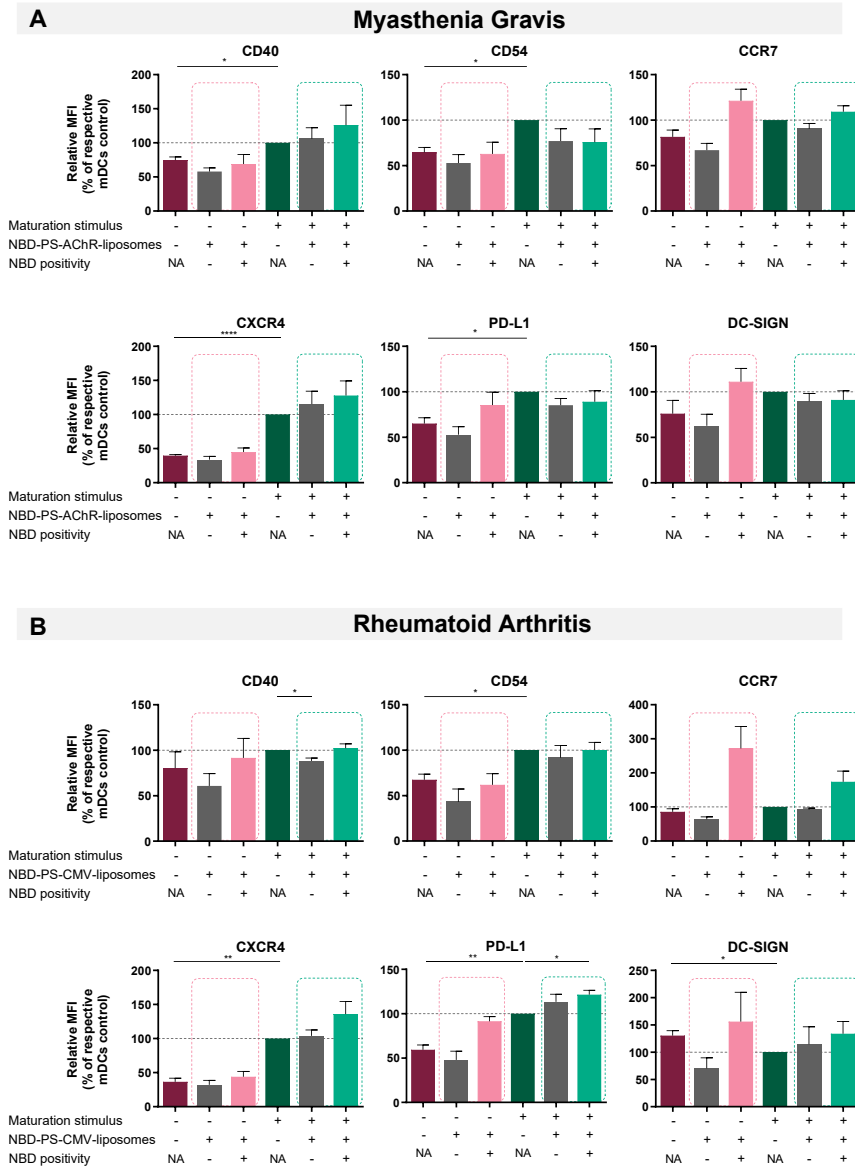


Figure 24. Expression of surface markers in DCs after NBD-PS-loaded-liposomes phagocytosis. (A) Relative MFI values of CD40, CD54, CCR7, CXCR4, PD-L1 and DC-SIGN in iDCs and mDCs obtained from MG (n=3-4) and **(B)** RA patients (n=3-4). Bars represent iDCs (dark pink), iDCs not positive for NBD fluorescence after NBD-PS-loaded-liposome coculture (grey), iDCs positive for NBD fluorescence after NBD-PS-loaded-liposome coculture (light pink), mDCs (dark green), mDCs not positive for NBD fluorescence after NBD-PS-loaded-liposome coculture (grey) or mDCs positive for NBD fluorescence after NBD-PS-loaded-liposome coculture (light green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing a culture condition with the mDCs control condition (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, One sample Wilcoxon signed-rank test).

8. MDCs that engulf PS-loaded-liposomes present the encapsulated peptide through MHC class II molecules

To determine whether DCs that engulf PS-loaded liposomes were capable of processing the cargo and presenting it via MHC class II molecules for subsequent interaction with T cells, a proteomic study was conducted. To this end, matured DCs from healthy controls were cocultured for 24 hours with PS-liposomes loaded with either the AChR extracellular domain or ADAMTS13 SPACER peptides. As control conditions, DCs were incubated with the peptides alone or left unstimulated.

Prior to proteomic analysis of the presented peptides, the differentiation efficiency of each condition was assessed, confirming the successful differentiation of monocytes into DCs, as evidenced by the high surface expression of CD11c across all conditions (**Figure 25A**). To validate the previously observed biological changes in DCs that internalized PS-liposomes, the expression of the PS receptors TIM-4 and CD36 was evaluated (**Figure 25B**). The results demonstrated an increased expression of these receptors in DCs treated with PS-liposomes compared to basal conditions, whereas no such increase was observed in conditions where only peptides were added.

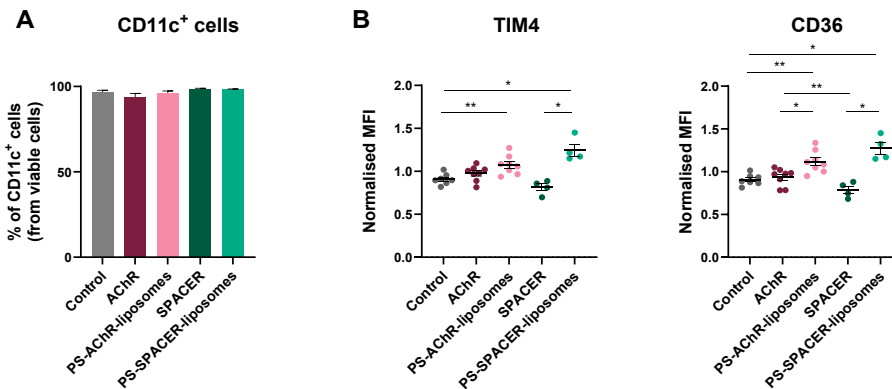


Figure 25. Monocytes from control subjects are efficiently differentiated to mDCs and after PS-loaded-liposomes coculture increase their PS receptors expression. (A) Percentage of CD11c⁺ cells after 5 days of differentiation and **(B)** normalised MFI of TIM4 and CD36 by dividing the value of each data point by the value of the pooled mean of the respective experiment (n= 4-8). Bars represent control condition (grey), mDCs cultured with AChR peptide (dark pink), mDCs cultured with PS-AChR-liposomes (light pink), mDCs cultured with SPACER peptide (dark green) and mDCS cultured with PS-SPACER-liposomes (light green). Data represented as mean $\pm$ SEM and normalised MFI. Significant differences were found when comparing among culture conditions (** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test).

Moreover, membrane expression of CD86, CD40, CD25 markers was evaluated; however, this time not showing significant differences (**Supplementary Figure 21**). Nevertheless, it is worth noting that for example HLA-DR expression tended to increase after PS-liposome coculture in both types of conditions always in comparison to basal DCs condition. In addition, conditions treated with SPACER and SPACER-loaded PS-liposomes, presented a decrease in CD40 expression, and an increase in CD14 expression. Regarding CD54 molecule, this time in contrast to previously seen, coculture of PS-liposomes tended to increase its expression. Accordingly with previous results, expression of CCR7 and CXCR4 statistically increased in conditions with loaded-PS-liposomes but not in those conditions with peptides alone or unstimulated.

To gain deeper insight into HLA class II antigen presentation, matured DCs preincubated with PS-loaded-liposomes, peptides, or left unstimulated were lysed, and HLA-DR and HLA-DQ molecules were purified using monoclonal antibodies conjugated to Sepharose. The peptides bound to the purified MHC class II molecules were dissociated and subsequently analysed by mass spectrometry. Additionally, each donor was genotyped to determine their HLA type, allowing for the identification of preferential peptide binding patterns associated with specific HLA alleles (**Supplementary Table 3**). This approach enabled the identification of a diverse repertoire of peptides, revealing that HLA-DR molecules presented a substantially higher number of peptides compared to HLA-DQ molecules (**Figure 26A and Supplementary Table 4**). Furthermore, immunopeptidome analysis of each control donor demonstrated donor-specific variability in the repertoire of peptides presented by HLA-DR molecules, as expected, highlighting the inherent diversity of human antigen presentation (**Supplementary Figure 22**). A comparative heatmap of the 50 most frequently presented proteins across donors showed a consistently high expression of proteins such as albumin, which has been widely reported in HLA-DR proteomic analyses (**Supplementary Figure 23**) [404]. Among all conditions, including unstimulated, peptide or loaded PS-liposome conditions, classification by protein class of the identified proteins using the Panther classification system revealed that among all presented peptides, most of them are known to be involved in physiological processes such as cytoskeletal organization, metabolite interconversion and enzymatic protein modifying (**Figure 26B**). Moreover, in **Supplementary Figure 24** we reflect common proteins presented among samples, worth noting that 7 physiological proteins such as albumin, heat shock protein, histone, prolow-density lipoprotein receptor-related protein or vimentin were constitutively presented through HLA-DR molecules in all conditions.

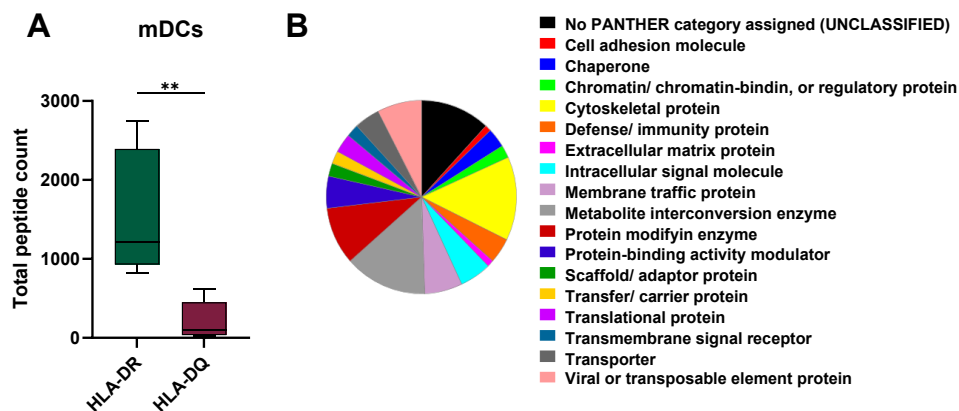


Figure 26. Peptide and protein classification presented by HLA class II molecules on basal mDCs. (A) Comparative of total peptide counts presented by HLA-DR and HLA-DQ molecules on mDCs studied by mass spectrometry (** $p < 0.01$, Wilcoxon matched-pairs signed rank test). **(B)** Pie chart supergrouping protein families by protein class of the HLA-DR ligandome using their GO term. The graph was generated using the web tool PANTHER (<https://pantherdb.org/>, accessed on 12 September 2024).

Focusing on matured DCs incubated with peptides from autoantigens or PS-loaded-liposomes, total peptide count presented by HLA class II molecules followed the same trend observed in unstimulated mDCs, with higher counts in HLA-DR molecules than HLA-DQ, in both AChR and SPACER peptide, encapsulated or not, conditions (**Figure 27 A and B**). Unfortunately, when mDCs were cultured with AChR peptide, this peptide was only identified in a few donors, presented by HLA-DR or HLA-DQ molecules (**Supplementary Table 5**), and when mDCs were cultured with PS-AChR-liposomes the peptide was barely detected in only one condition, presented by HLA-DR from donor 8 (**Table 16**). Regarding mDCs cultured with either SPACER peptide and PS-SPACER-liposomes, the specific peptide was successfully detected in all conditions at medium/high confidence (**Supplementary Table 6 and Table 17**).

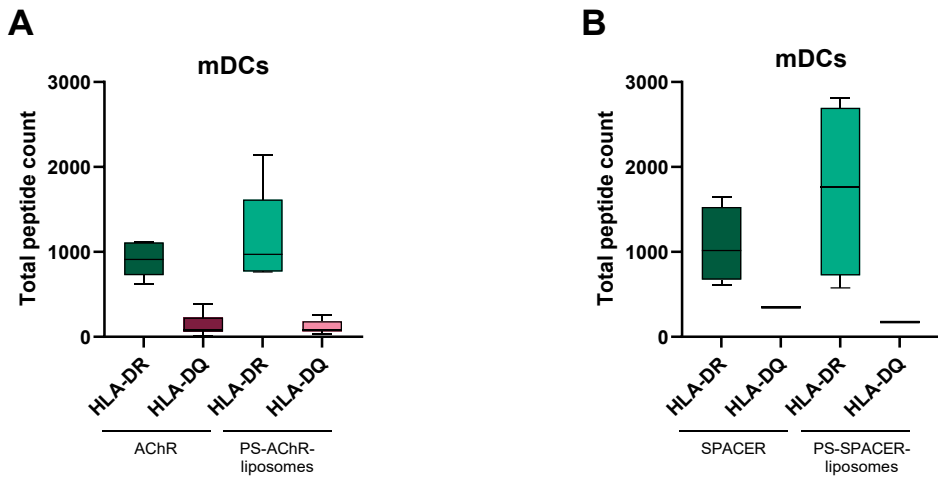


Figure 27. Total peptide counts of the immunopeptidome presented by HLA class II molecules on mDCs cultured with free peptides or PS-loaded-liposomes studied by mass spectrometry. **(A)** Total peptide counts in mDCs cultured with free AChR peptide or PS-AChR-liposomes. **(B)** Total peptide counts in mDCs cultured with free SPACER peptide or PS-SPACER-liposomes.

Table 16. Summary of the number peptides presented by HLA class II molecules identified in PS-AChR-liposome conditions

HLA-DR					HLA-DQ				
D	Nº of proteins	Nº of peptides	Nº of AChR peptides		D	Nº of proteins	Nº of peptides	Nº of AChR peptides	
			High	Low				High	Low
1	217	892	ND	ND	1	41	79	ND	ND
2	266	1048	ND	ND	2	40	63	ND	ND
4	207	775	ND	ND	4	27	39	ND	ND
6	303	1072	ND	ND	5	72	254	ND	ND
7	222	768	ND	ND	6	47	119	ND	ND
8	421	1799	1	ND	7	39	71	ND	ND
9	437	2144	ND	ND	8	84	184	ND	ND
10	201	764	ND	ND					

D: donor; ND: not detected

Table 17. Summary of the number peptides presented by HLA class II molecules identified in PS-SPACER-liposome conditions

HLA-DR					HLA-DQ				
D	Nº of proteins	Nº of peptides	Nº of SPACER peptides		D	Nº of proteins	Nº of peptides	Nº of SPACER peptides	
			High Medium	Low				High Medium	Low
6	201	575	16	ND	10	45	174	59	ND
8	332	1174	5	ND					
9	482	2359	22	ND					
10	337	2810	38	ND					

D: donor; ND: not detected

9. Phagocytosis of PS-liposomes by DCs from MG and AR patients impairs their ability to stimulate autologous T cell proliferation

Once, the ability of human monocyte derived APCs to uptake loaded-PS-liposomes and present the encapsulated peptide through HLA class II molecules, next step was to check their ability to induce autologous T cell proliferation. To this end, proliferation was tested by co-culture of different conditions of MG and RA monocyte-derived DCs pulsed with the corresponding peptide. These cells were cultured together with the corresponding PS-loaded-liposomes or empty PS-liposomes for 24h, and subsequently with autologous CD14⁺ CTV-stained PBMC for 6 days, as shown in **Figure 28**. To perform this study MG patient number 4 and 7 were excluded and classified as non-responders, correlating with the haplotype of HLA alleles DQA1*03 and DQB1*03 (**Supplementary Table 1**). As expected, in both diseases, the proliferation of CD4⁺ T cells induced by mDCs was consistently higher than that induced by iDCs condition ($p<0.0001$) (**Figure 28**). Furthermore, proliferation induced by iDCs cocultured with loaded PS-liposomes and empty PS-liposomes was comparable to that observed in the basal iDCs conditions with no statistically significant differences. Notably, in the presence of PS-loaded-liposomes, mDCs showed a decrease in CD4⁺ T cell proliferation in comparison to mDCs alone in both diseases, with this reduction reaching statistical significance in MG and RA patients ($p<0.001$). Interestingly, those mDCs cultured with empty PS-liposomes, although presenting a tendency to decrease CD4⁺ T cell proliferation, did not impair the autologous proliferation as efficiently as loaded PS-liposomes. Regarding the

CD8⁺ T cell proliferation, that induced by the mDCs, as expected, was consistently higher than that induced by the iDCs, in both MG and RA patients ($p<0.01$ and $p<0.001$, respectively). Additionally, iDCs with loaded or empty PS-liposomes maintained low proliferation rates. When mDCs were cocultured with PS-loaded-liposomes, the ability to induce CD8⁺ T cell proliferation was also statistically reduced in comparison to mature basal DCs conditions in both diseases ($p<0.05$ and $p<0.001$ respectively), while PS-empty-liposomes did not present this statistical reduction.

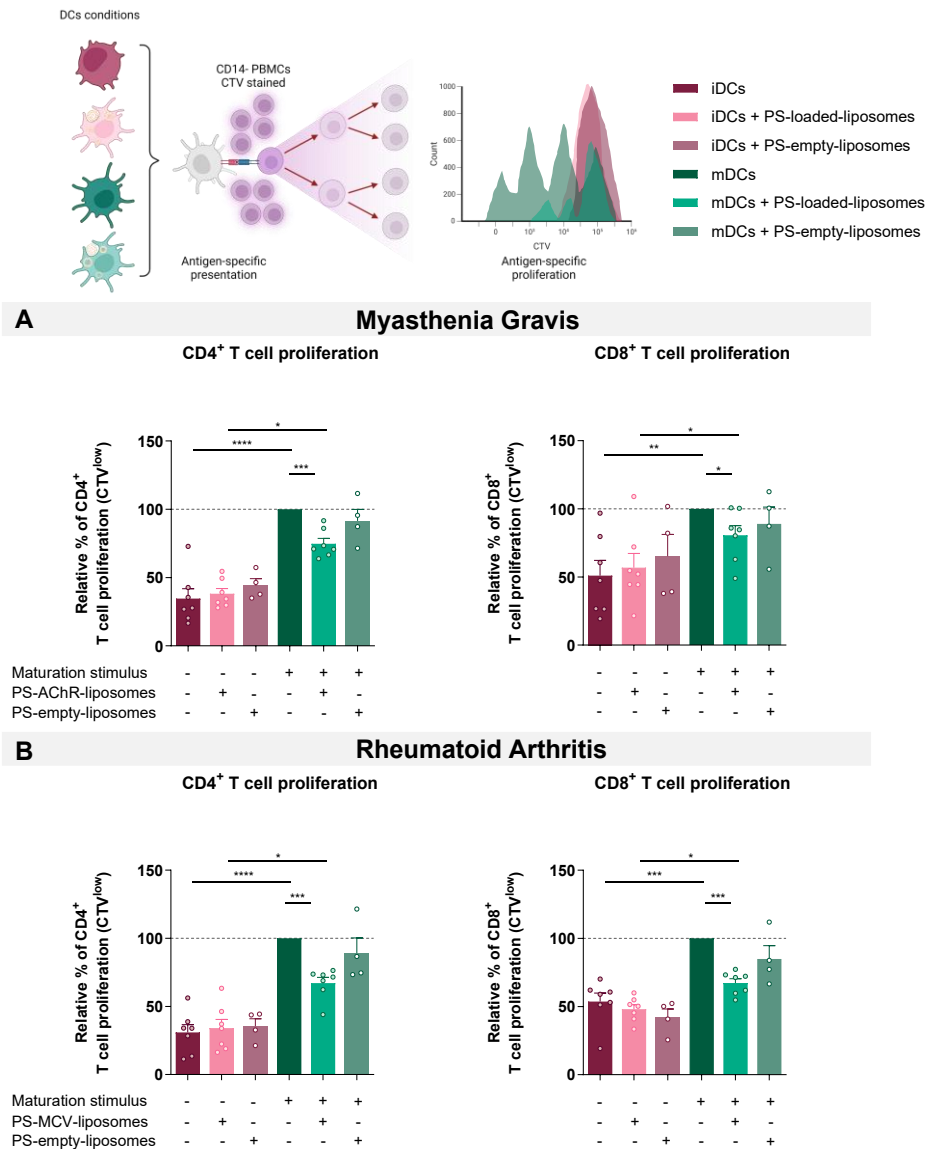


Figure 28. PS-loaded-liposomes phagocytosis impairs the ability of DCs to induce autologous T cell proliferation. (A) Autologous proliferation of CD4⁺ and CD8⁺ T cells induced by DCs cultured with autoantigen from MG (n=4-7) and (B) RA patients (n=4-7). Bars represent iDCs (dark pink), iDCs after PS-loaded-liposome phagocytosis (light pink), iDCs after empty PS-liposome phagocytosis (purple), mDCs (dark green), mDCs after PS-loaded-liposome phagocytosis (light green) or mDCs after empty PS-liposome phagocytosis (greyish-green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions (***p < 0.001, **p < 0.01, *p $\leq$ 0.05, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition (***p < 0.0001, ***p < 0.001, **p < 0.01, *p $\leq$ 0.05, One sample Wilcoxon signed-rank test).

10. CD14⁺ cells from SFMCs exhibit phenotypic modulation following PS-liposome uptake

Despite the limited availability of synovial fluid from RA patients, where possible, SFMCs were isolated. The immune cell subset composition and the gating strategy followed to analyse whole SFMCs is reflected in **Supplementary Figure 25A** and **Supplementary Figure 26**, respectively. To investigate the effect of PS-MCV-liposome uptake on these, relatively understudied cells, the CD14⁺ fraction was isolated and cocultured under identical conditions as monocytes for 5 days. PS-MCV-liposomes were added to the media at fifth day and the phenotype was assessed 24 hours later. On day 6, CD11c expression was evaluated, showing consistently high percentages of positive cells, correlating with a good differentiation into synovial fluid derived DCs (**Supplementary Figure 25B**).

As expected, the expression levels of TIM4 and CD36 PS receptors tended to be higher on iDCs compared to their respective mature control condition, although it did not reach the statistical significance (**Figure 29**). Notably, the expression of these receptors also tended to be increased when iDCs were cocultured with PS-MCV-liposomes. MDCs also presented a similar trend of increased PS receptor expression following the uptake of PS-MCV-liposomes. In contrast, the expression of the Integrin $\alpha\beta 5$ marker remained constant in mature conditions, with no significant differences observed.

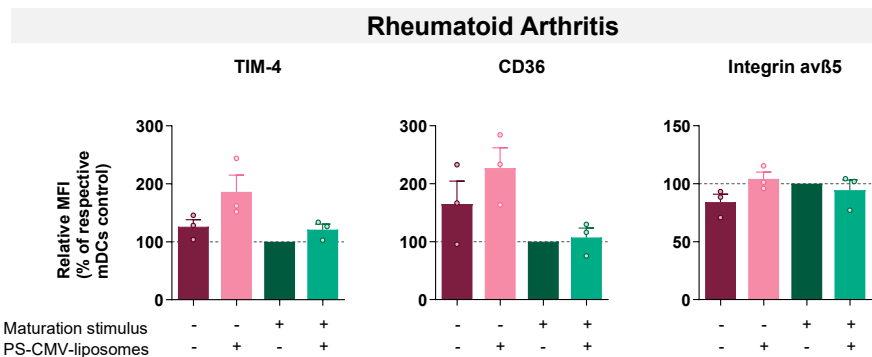


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Figure 29. PS-receptors expression is modulated in synovial fluid derived DCs after PS-MCV-liposomes phagocytosis. Relative MFI of TIM-4, CD36 and Integrin $\alpha\beta 5$ membrane expression in synovial fluid derived iDCs and mDCs obtained from RA patients (n=3). Bars represent iDCs (dark pink), iDCs after PS-MCV-liposomes phagocytosis (light pink), mDCs (dark green) or mDCs after PS-MCV-liposomes phagocytosis (light green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions (** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition (** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, One sample Wilcoxon signed-rank test).

Regarding the antigen presentation molecules (**Figure 30**), HLA-DR marker showed a statistically significant lower expression in iDCs conditions when compared to mDCs control conditions ($p < 0.01$), and HLA-ABC showed the same trend although non-significant. Interestingly, PS-MCV-liposomes appeared to enhance the expression of these markers, being statistically significant in the immature conditions, in both HLA-DR and HLA-ABC markers ($p < 0.05$). However, in mDCs, the increase in marker expression following PS-MCV-liposome uptake did not reach statistical significance. The expression levels of CD40 and CD54 were consistently lower in the immature control conditions compared to the mature condition ($p < 0.0001$ and $p < 0.05$, respectively). Similarly, CD86 and CD25 expression followed this trend although without reaching statistical significance. Coculture with PS-liposomes resulted in a slight, but not statistically significant, increase in CD40 and CD68 expression levels. Interestingly mature cells cocultured with PS-MCV-liposomes presented a similar trend observed in monocyte-derived DCs, with a decrease in CD54 expression levels, although this was also not statistically significant. CCR7 and CXCR4 chemokine receptors notably displayed a tendency to increased membrane expression after the PS-MCV-liposomes coculture in both immature and mature conditions. Furthermore, matching the trend observed in mature monocyte-derived DCs, PS-MCV-liposomes resulted in an almost statistically significant increase in CXCR4 expression in mDCS ($p = 0.056$). CD14 expression levels were similar across all conditions, however, there was a slight increase observed in those conditions cocultured with PS-MCV-liposomes. DC-SIGN also showed this tendency, but this was only apparent in the immature conditions. PD-L1 consistently showed a higher expression in those mature conditions compared to immature ones ($p < 0.001$). Moreover, phagocytosis of PS-MCV-liposomes led to a notable increase in PD-L1 expression level in both immature and mature conditions, with the mature condition showing a statistically significant increase when compared to the control mature condition ($p < 0.01$).

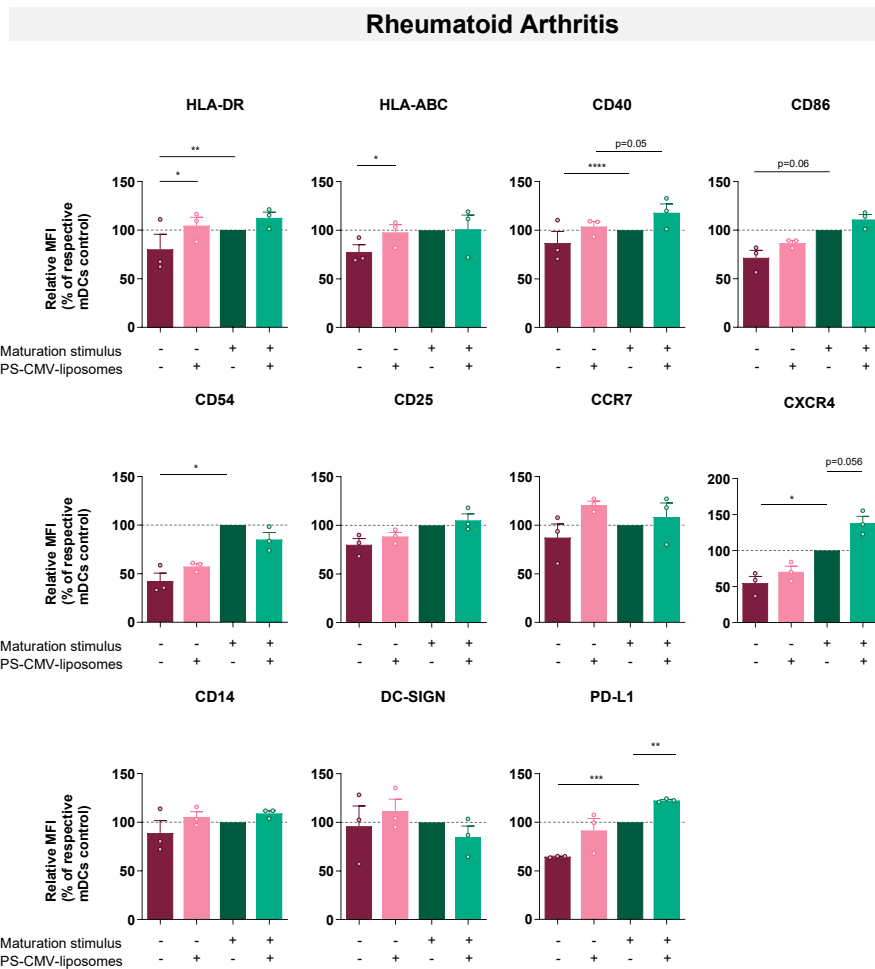


Figure 30. Membrane markers are modulated in synovial fluid derived DCs after PS-MCV-liposomes phagocytosis. Relative MFI values of HLA-DR, HLA-ABC, CD40, CD86, CD54, CD25, CCR7, CXCR4, CD14, DC-SIGN and PD-L1 membrane expression in synovial fluid derived iDCs and mDCs obtained from RA patients (n=3). Bars represent iDCs (dark pink), iDCs after PS-MCV-liposomes phagocytosis (light pink), mDCs (dark green) or mDCs after PS-MCV-liposomes phagocytosis (light green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions (** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition (** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, One sample Wilcoxon signed-rank test).

11. SFMCs CD14⁺ cells after the PS-liposome uptake present impaired ability to stimulate autologous T cell proliferation from the synovial fluid

With the aim to assess the effect of PS-MCV-liposomes on tolerogenic ability of the synovial fluid monocyte derived DCs, a coculture with CTV-stained autologous CD14⁺ SFMCs fraction was performed for 6 days, as done before with the CD14⁺ fraction of PBMCs. In the proliferation of CD4⁺ T cells, basal iDCs and iDCs engulfing PS-MCV-liposomes conditions tended to present lower proliferation rates in comparison to mDCs conditions, in this case, not showing statistically differences between the corresponding mature condition (**Figure 31**). However, in the CD8⁺ T cell compartment these conditions could not impair T cell proliferation, presenting unusually high proliferation values. Interestingly, in both, CD4⁺ and CD8⁺ compartments, mDCs phagocytosing PS-MCV-liposomes impaired autologous T cell proliferation in comparison to basal mDCs conditions, reaching statistical significance in CD8⁺ T cell compartment ($p < 0.05$).

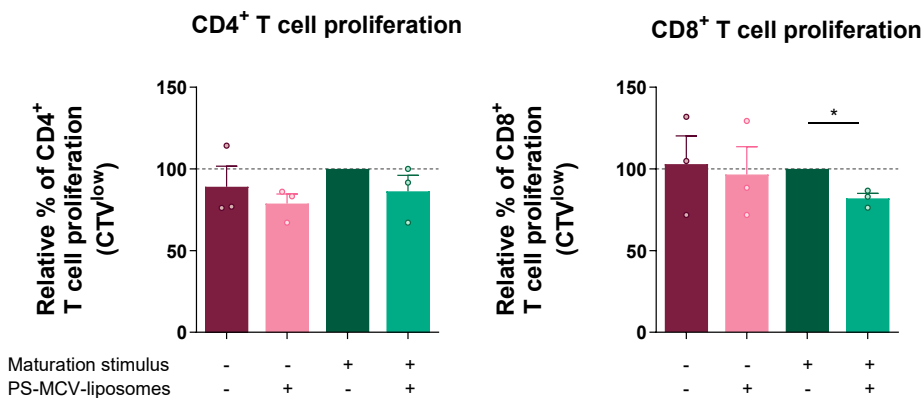


Figure 31. Autologous T cell proliferation of SFMCs after coculture with MCV peptide pulsed synovial fluid monocyte derived DCs which phagocytosed PS-MCV-liposomes (n=3). Bars represent iDCs (dark pink), iDCs after PS-loaded-liposome phagocytosis (light pink), mDCs (dark green) or mDCs after PS-loaded-liposome phagocytosis (light green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions (* $p \leq 0.05$, One sample Wilcoxon signed-rank test).

12. PS-liposomes are capable to interact with B cells

To further investigate the interaction of PS-loaded-liposomes with other APCs and given prior observations of PS-loaded-liposomes interaction with B cells, we cultured freshly isolated PBMCs from healthy controls with fluorescently NBD-labelled PS-Ins-liposomes [405]. Although the intention was to study B cells, we opted for a culture of total PBMCs since when these cells are isolated and cultured separately for 24h, their viability decreases markedly (data not shown). As expected, monocytes exhibited the highest capacity for phagocytosing, with $79.60 \pm 9.05\%$ (Mean $\pm$ SD) of NBD positivity after 24 hours of coculture (**Supplementary Figure 27**). Despite their low frequency in circulation, DCs also demonstrated a notable uptake of PS-loaded-liposomes, with $78.6 \pm 8.88\%$ (Mean $\pm$ SD) positivity, reinforcing their role in AC clearance and antigen processing. Interestingly, B cells emerged as a subset capable of interacting with PS-liposomes, as $3.20 \pm 1.33\%$ (Mean $\pm$ SD) of total B cells were positive for the NBD fluorochrome when incubated with NBD-labelled PS-loaded-liposomes, either with insulin or AChR peptide, and being statistically significant in comparison to control B cells incubated with PBS ($p < 0.01$) (**Figure 32A and B**).

To validate these findings in autoimmune disease settings, cryopreserved CD14⁻ PBMC fractions from MG and RA patients—comprising primarily T cells, B cells, and NK cells—were thawed and cultured with the corresponding NBD-labelled PS-loaded liposomes. After 24 hours of incubation, B cell interactions with PS-liposomes in both MG and RA patient samples displayed comparable percentages to those observed in healthy controls, further supporting the robustness and consistency of these observations across different immune conditions ($3.12 \pm 3.77\%$ and $3.98 \pm 3.91\%$, respectively) (Mean $\pm$ SD) (**Figure 32C**).

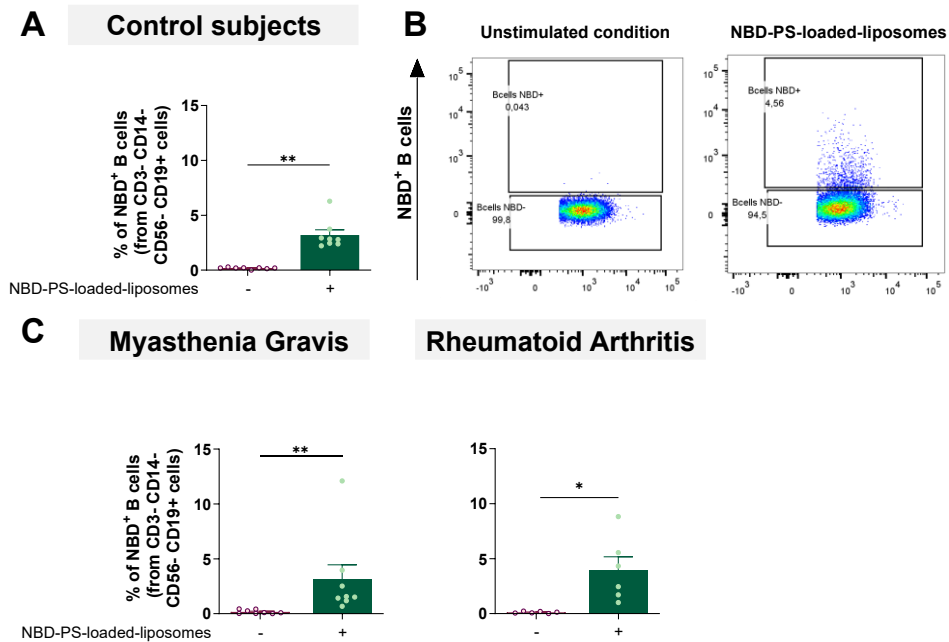


Figure 32. B cells following a 24-hour incubation with fluorescently NBD-labelled PS-loaded-liposomes exhibit positivity for the NBD fluorochrome. (A) Comparative of NBD positive B cells from PBMCs of control subjects incubated or not with NBD-PS-loaded-liposomes (n=8). **(B)** Representative gating strategy used to analyse NBD⁺ cells. **(C)** Comparative of NBD positive B cells from CD14⁻ PBMCs of MG and RA patients incubated or not with NBD-PS-loaded-liposomes (n=9 and n=6, respectively). B cells defined as CD3⁺ CD14⁻ CD11c⁻ CD19⁺ cells. Bars represent unstimulated B cells (dark pink) or B cells incubated with NBD-labelled PS-loaded-liposomes (dark green). Data represented as mean ± SEM. Significant differences were found when comparing among conditions (***p< 0.001, **p<0.01, Wilcoxon matched-pairs signed rank test).

13. B cells capable of interacting with PS-loaded-liposomes undergo modulation toward a tolerogenic profile

To investigate how the interaction of B lymphocytes with PS-loaded liposomes influences functionality, the CD14⁻ fraction of PBMCs from MG and RA patients was incubated with NBD fluorescently labelled PS-liposomes, and the production of IL-10 and TGF- β was assessed in B cell subsets that interacted with the liposomes (NBD⁺) versus those that did not (NBD⁻). As a control condition, CD14⁻ PBMCs incubated with PBS were included in the analysis. Notably, B cells from MG patients that interacted with PS-AChR-liposomes exhibited a statistically significant increase in IL-10 and TGF- β production compared to both the basal condition and the NBD⁻ B cell population ($p < 0.001$ and $p < 0.01$, respectively) (**Figure 33A**). Similarly, B cells from RA patients displayed the same behaviour, with enhanced production of these anti-inflammatory cytokines following interaction with PS-MCV-liposomes ($p < 0.01$ and $p < 0.05$), comparing with both basal and NBD⁻ B cells, respectively (**Figure 33B**). These findings suggest that PS-liposome engagement may promote an immunoregulatory phenotype in B cells, highlighting a potential mechanism by which PS-liposomes can modulate their immune responses.

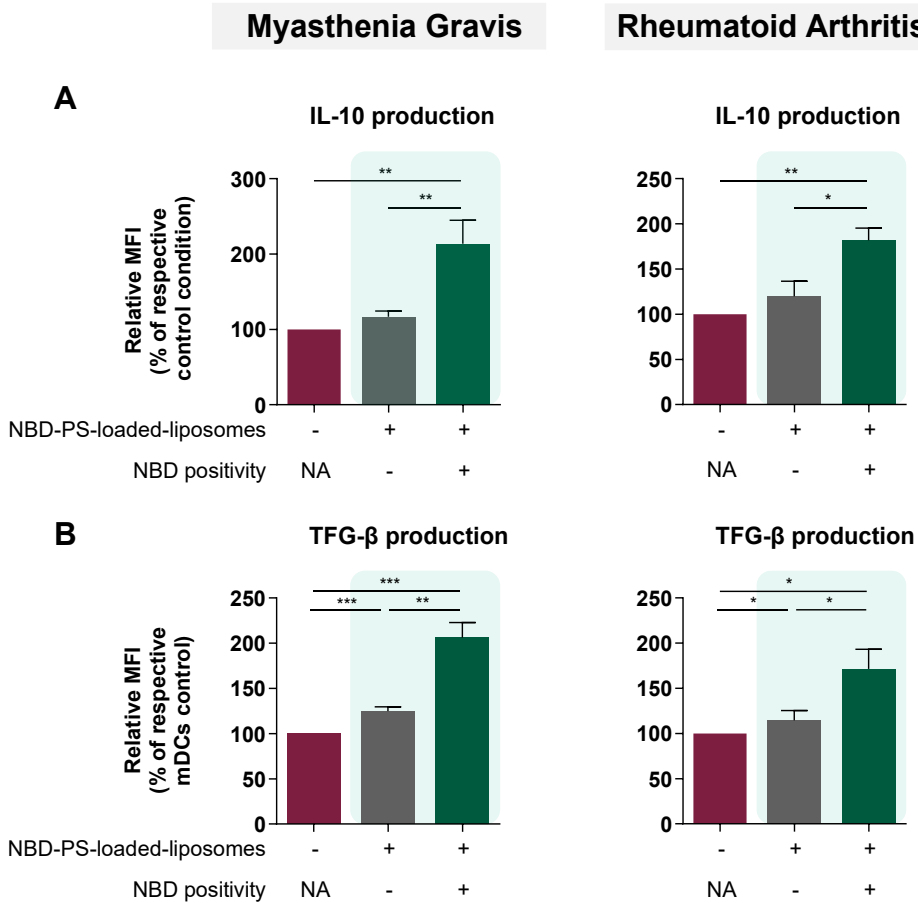


Figure 33. CD19⁺ cells from MG and RA patients upregulate IL-10 and TGF-β production after PS-loaded-liposomes interaction (n=9 and n=6, respectively). (A) Relative MFI levels of IL-10 production in B cells from both MG (left panel) and RA patients (right panel). (B) Relative MFI levels TGF-β production in B cells from both MG (left panel) and RA patients (right panel). B cells defined as CD3⁺CD14⁺CD11c⁺CD19⁺ cells. Bars represent control condition (dark pink), B cells negative for the NBD fluorescence of the PS-loaded-liposomes (grey) or B cells positive for the NBD fluorescence of the PS-loaded-liposomes (dark green). Data represented as mean ± SEM of relative MFI, with the MFI of each culture condition normalized to the control condition. Significant differences were found when comparing among interacting or not B cells with PS-loaded-liposomes (**p<0.01, *p ≤0.05, Wilcoxon matched-pairs signed rank test) and when comparing a condition with the control condition (***p<0.001, **p ≤0.01, One sample Wilcoxon signed-rank test).

14. B cells can internalize PS-liposomes labelled with pHrodo-dye

To gain further insight into the nature of the interaction between B cells and PS-liposomes, we employed the pH-dependent fluorochrome pHrodo. As previously described, PS-liposomes labelled with this fluorophore emit fluorescence only in acidic environments (pH < 4), such as those found in lysosomes. Based on this property, fluorescence is expected to be detected exclusively when PS-liposomes have been internalized and processed within the endo-lysosomal compartment. Following a 24-hour incubation of the CD14⁺ fraction of PBMCs, fluorescence was assessed across different immune cell subsets (**Figure 34**). As expected, monocytes and DCs presented the highest rates of phagocytosis, with $96.9 \pm 2.67\%$ and $73.41 \pm 10.29\%$ of positivity, respectively. Remarkably, B cells also displayed a measurable fluorescence signal, with $5.02 \pm 2.79\%$ positivity, confirming their capacity to actively internalize PS-liposomes. Probably, the same subset capable of interact with NBD-PS-loaded-liposomes.

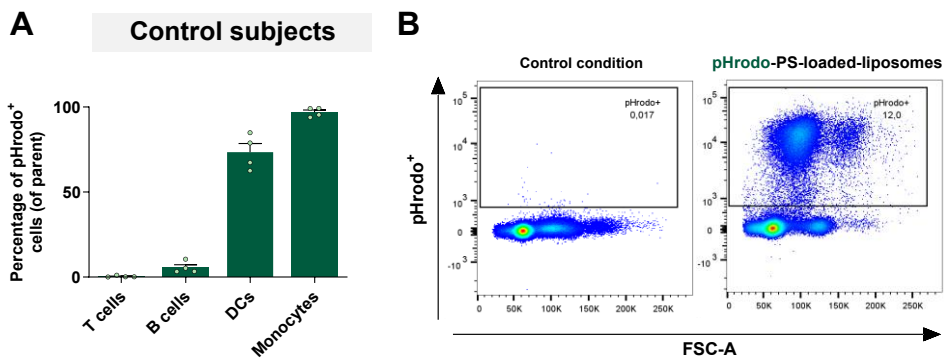


Figure 34. pHrodo positive cell subsets from healthy donors after the incubation for 24h with pHrodo-labelled PS-liposomes from (n=4). (A) Percentage of pHrodo⁺ cells in every PBMCs immune cell subsets. Bars represent conditions incubated with pHrodo-PS-AChR-liposomes (dark green). T cells defined as CD11c⁺ CD3⁺ CD19⁺ CD14⁺ cells; B cells defined as CD3⁺ CD14⁺ CD11c⁺ CD19⁺ cells; DCs defined as CD3⁺ CD19⁺ CD14⁺ CD11c⁺ and monocytes defined as CD3⁺ CD10⁺ CD11c⁺ CD14⁺ cells. Data represented as mean $\pm$ SEM. (B) Representative gating of immune cell subsets within PBMCs cultured with PBS or pHrodo-labelled PS-liposomes.

15. B cells express phosphatidylserine receptors on their surface

With the aim to further investigate the mechanisms by which B cells internalize PS-liposomes, we hypothesized that the uptake could be mediated by PS recognition receptors. To date, the expression of the PS receptor on B cells has been rather poorly studied. For this reason, PBMCs from healthy donors were incubated for 24 hours with either non-fluorescent or NBD-labelled PS-AChR-liposomes. Cells treated with PBS were used as the control condition. Following incubation, a panel of known PS receptors was assessed by flow cytometry to evaluate their expression profiles across different immune subsets. As expected, monocytes presented significantly higher mean MFI values for all PS receptors analysed when compared to B cells (data not shown). Among all the receptors, TIM-1, CD36, and Axl stood out as the most abundantly expressed PS receptors on monocytes (**Figure 35A**). Despite their generally lower MFI values, B cells were also found to express PS receptors, with Axl showing the highest expression, followed by integrin $\alpha\beta v5$. Notably, Axl expression was significantly higher than that of CD36 and TIM-4 on B cells (**Figure 35B**). Interestingly, a comparative analysis between PBS-treated cells and those exposed to PS-AChR-liposomes revealed a statistically significant downregulation of Axl expression in both monocytes and B cells ($p < 0.0001$ and $p < 0.01$, respectively), while the expression levels of the rest of PS receptors remained relatively stable (**Figure 35C**). Furthermore, within the population of cells cocultured with NBD-labelled PS-AChR-liposomes, monocytes that internalized the liposomes (NBD⁺) generally exhibited higher levels of PS receptor expression compared to their NBD⁻ counterparts. However, Axl expression was notably reduced in the NBD⁺ monocytes ($p < 0.01$), suggesting a receptor downregulation upon liposome uptake (**Figure 35D**). A similar trend was observed in B cells, where NBD⁺ cells displayed elevated expression of all assessed PS receptors relative to NBD⁻ cells, with statistically significant increases in integrin $\alpha\beta v5$, TIM-1, TIM-3, and Axl ($p < 0.00001$ and $p < 0.001$, respectively).

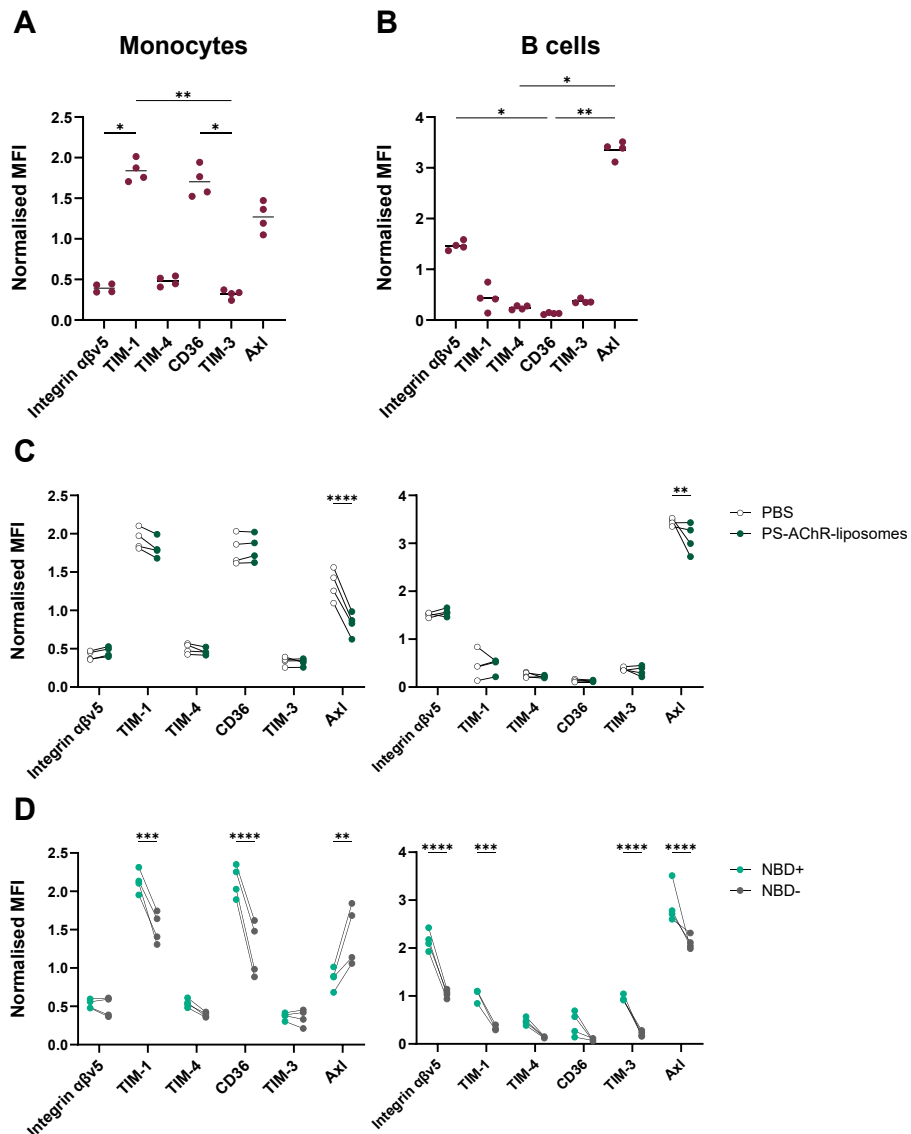


Figure 35. Expression of Integrin $\alpha\beta v5$, TIM-1, TIM-4, CD36, TIM-3 and Axl PS receptors on monocytes and B cells from PBMCs of healthy donors after coculture with PS-AChR-liposome or NBD-labelled PS-AChR-liposomes (n=4). (A) Basal expression levels of PS receptors on monocytes and B cells. (B) Comparative expression levels of PS receptors on monocytes and B cells when left unstimulated or cocultured with PS-AChR-liposomes. (C) Comparative expression levels of PS receptors on NBD positive or negative monocytes and B cells cocultured with NBD-labelled PS-AChR-liposomes. Monocytes defined as CD19⁻ CD11c⁻ CD14⁺ cells; B cells defined as CD14⁻ CD11c⁻ CD19⁺ cells. Data represented as normalised MFI, by dividing the value of each data point by the value of the pooled mean of the respective experiment. Significant differences were found when comparing among PS receptors from a

same group (**p<0.01, *p ≤0.05, Kruskal-Wallis test) or between two groups from the same subset (****p<0.0001, ***p<0.001, **p<0.01, *p ≤0.05, ordinary two-way ANOVA).

SECTION IV. Regulatory evaluation of PS-AChR-liposomes safety

Given that in the future clinical trials PS-liposomes are intended to be administered intravenously, it is essential to assess a range of parameters to ensure their safety upon contact with human blood. To this end, a comprehensive evaluation was performed to address potential risks related to haemostatic balance, hemocompatibility, and inflammatory responses. Specifically, coagulation parameters, osmotic fragility, and the potential to induce CRS were systematically evaluated. Since MG represents the primary therapeutic target of the current development pipeline, PS-liposomes loaded with the AChR peptide (PS-AChR-liposomes) were selected for all safety-related assessments. In addition to these safety studies, and in alignment with regulatory requirements, a functional potency assay was developed and validated. This assay is a critical component of the regulatory dossier, as it ensures consistent therapeutic efficacy and serves as a batch-release criterion. Consequently, the potency assay is presented in this chapter alongside other preclinical evaluations relevant for regulatory submission.

1. PS-AChR-liposomes are successfully produced

PS-liposomes were formulated with a defined molar ratio of DOPS/DMPC/CH to mimic apoptotic bodies, presenting PS on their surface. **Table 18** shows the physicochemical features of the PS-liposomes used in **SECTION IV**. To ensure efficient phagocytosis, the PS-liposomes were consistently designed with a minimum diameter of 500 nm and maintained at a concentration of approximately 30 mM. The Z-potential was confirmed to be negative, ensuring their tolerogenic potential.

Table 18. Physicochemical features of PS-liposomes used in SECTION IV

Liposome nomenclature	Number of batches	Lipid concentration (mM)	Diameter size (nm)	PdI	Z-potential (mV)	Encapsulation efficiency (%)
PS-AChR-liposomes	3	34.60 ± 7.95	555.57 ± 184.23	0.25 ± 0.04	-20.57 ± 1.39	46.38 ± 1.54

Data presented as mean ± SD; NA: not applicable; PdI: polydispersity index.

2. PS-AChR-liposomes do not alter hemocompatibility parameters of human blood

To determine whether PS-liposomes have an immediate impact on blood quality parameters, PS-AChR liposomes were directly incubated with blood of different 6 control subjects. All subjects in the present study met the exclusion and inclusion criteria and gave informed consent to participate. **Table 19** summarises their clinical data. The different concentrations of PS-AChR-liposomes were chosen to simulate the concentration reached in the bloodstream of a human after the IV administration (0.18mM), seven times more concentrated (1.4mM) and finally, fifteen times more concentrated (2.8mM). After 5-, 30- and 120-minutes incubation, the erythrocyte aggregation, haemoglobin oxidation and osmotic fragility were evaluated to detect possible alterations in those blood quality parameters.

Table 19. Data from control subjects included in the blood parameter test

Parameter	Control subjects
N	6
Gender	3/6 (50%) Female 3/6 (50%) Male
Age (years)	34.00±6.96

Data presented as mean±SD.

First, the morphological profile of each volunteer' erythrocytes was assessed, being always within the normal range for their sex and age (data not shown). Subsequently, single cell analysis of the red blood cells was performed in order to determine the erythrocyte aggregation (**Figure 36A**). In case of aggregation, a significant decrease in RBC (Red Blood Cells) would be expected, as clots would be produced. In this case, single cell counts in the control group (no PS-AChR-liposomes added), as expected, proved to be consistent over time, 5 minutes after incubation with PBS presented a mean of 6986.36 ± 29.75 RBCs/ μ l; 30 minutes after 6839.45 ± 247.92 RBCs/ μ l and 120 minutes after 6600.80 ± 592.98 RBCs/ μ l. Similar values were obtained with the blood incubated with the highest concentration (2.8 mM) after 5 minutes with a mean of 6665.02 ± 820.20 RBCs/ μ l; 7356.62 ± 1442.24 RBCs/ μ l after 30 minutes and 7284.91 ± 1265 RBCs/ μ l. The group incubated with 1.4 mM of PS-AChR-liposomes showed after 5 minutes a mean of 7361.98 ± 1039.35 RBCs/ μ l; 30 minutes timepoint

7013.12±1625.9 RBCs/μl and 120 minutes timepoint a mean of 7044.71±1905.16 RBCs/μl. Finally, the conditions cultured with the minimum concentration (0.18 mM) showed a mean of 6819.04±1130.54 RBCs/μl after 5 minutes; 6742.478±1397.9 RBCs/μl after 30 minutes and 7342.69±2017.26 RBCs/μl after 120 minutes. None of the different concentrations or time points presented statistically significant differences from each other.

The haemoglobin oxidation was measured by analysing the violet SSC absorption at 413nm (**Figure 36B**), in the case of the control conditions, all the timepoints showed similar values, 5 minutes after PBS incubation the mean of the MFI resulted to be 4752.50±197.28; after 30 minutes 5058.33±585.16 and after 120 minutes 5047.00±817.42. Conditions with PS-AChR-liposomes demonstrated to maintain similar MFI values among conditions and over time. Blood incubated with the highest concentration (2.8 mM) presented similar values being 5 minutes after 4983.50±503.94; 30 minutes 5149.5±609.57 and 120 minutes after 4917.17±784.94. The group incubated with 1.4 mM of PS-AChR-liposomes showed after 5 minutes a mean of 5264.17±890.40; after 30 minutes 5226.50±761.26 and after 120 minutes 5008.17±590.26. Finally, conditions cultured with the lowest concentration (0.18 mM) presented a mean of 4867.83±631.40 after 5 minutes incubation; 5075.50±686.49 after 30 minutes and 4985.33±587.26 after 120 minutes.

To test the resistance of the red blood cells to osmotic stress following incubation with PS-AChR-liposomes, a hypotonic solution of 0.55% NaCl was added to induce erythrocyte swelling due to water influx. The osmotic fragility of each condition was assessed by analysing light scatter properties, which allowed for monitoring erythrocyte lysis over time. The graphs in **Figure 37A** show how the erythrocytes start to burst following the hypotonic solution addition in donor 1, demonstrating the similarity to the control condition in each condition. To statistically validate this observation, the area under the curve (AUC) was calculated for each donor. **Figure 37B** demonstrates that there were no statistically significant differences among the groups, regardless of the incubation time with PS-liposomes or the concentration of used, in line with the previous results, showing no toxicity of PS-AChR-liposomes.

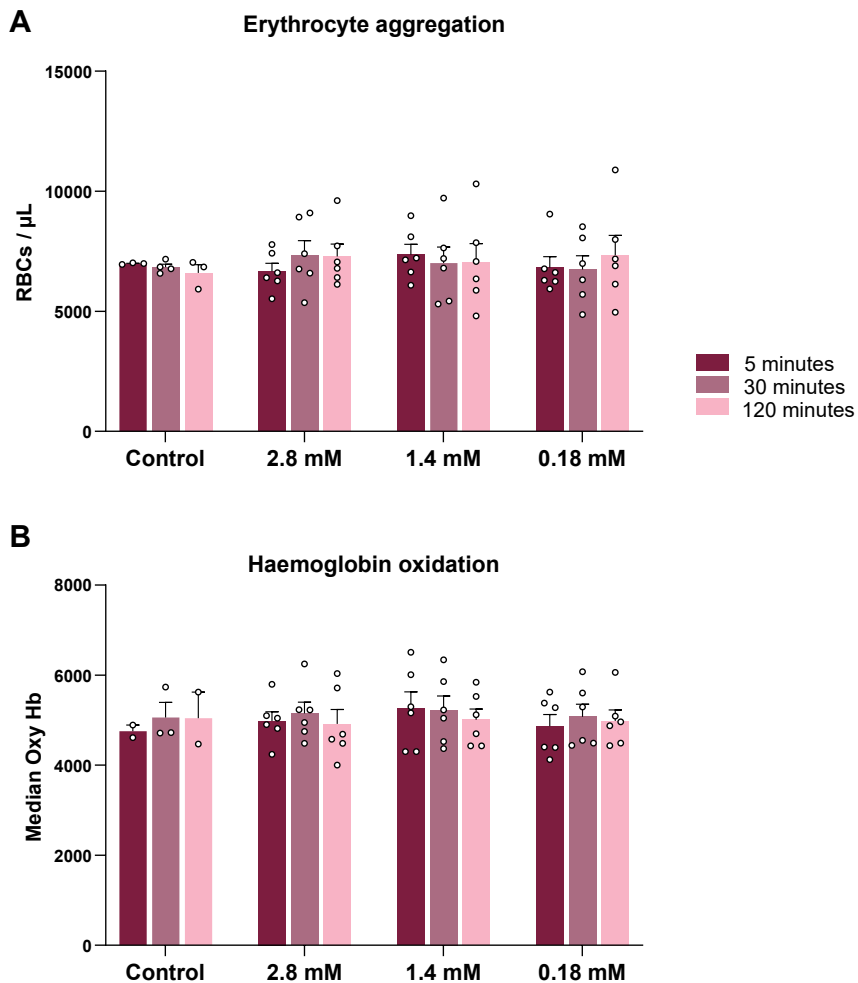


Figure 36. Blood levels of erythrocyte aggregation and haemoglobin oxidation after the incubation with PS-AChR-liposomes (n=6). (A) Red blood cell counts and (B) oxyhaemoglobin absorption after incubation with different concentrations of PS-AChR-liposomes at different timepoints. Bars represent 5 minutes timepoint (dark pink), 30 minutes timepoint (medium pink) and 120 minutes timepoint (light pink) and dots represent each donor. Results are represented as events/ μL or Median Fluorescence Intensity of violet SSC. Data represented as mean $\pm$ SEM. No significant differences were found when comparing among different conditions (Mixed-effects model (REML) with Tukey's multiple comparisons test).

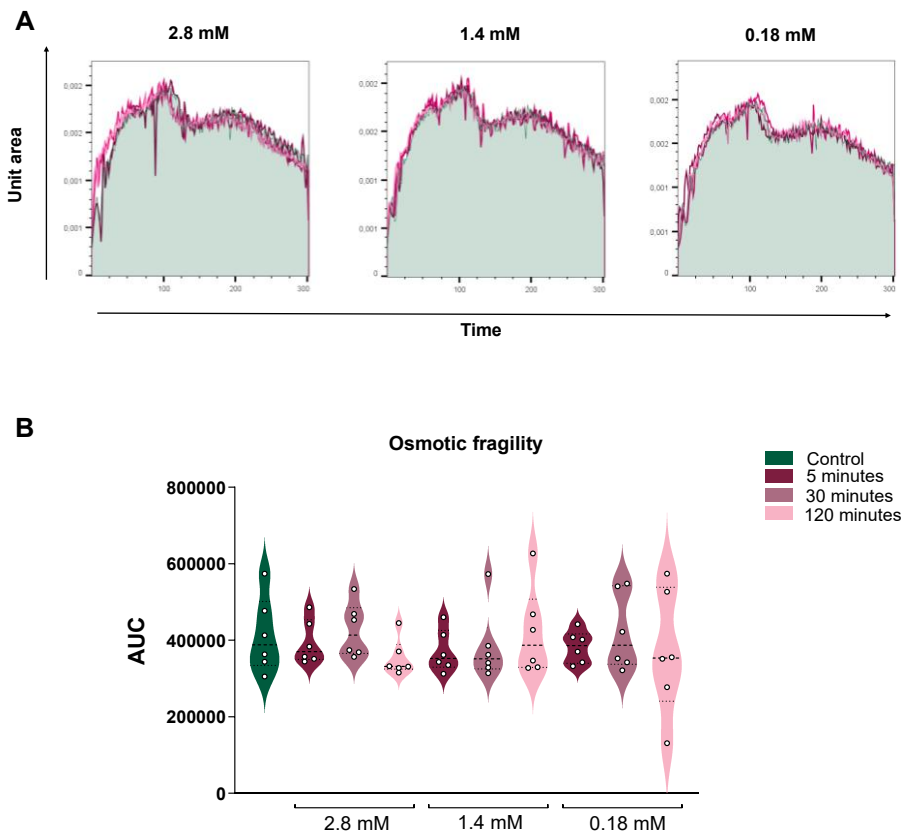


Figure 37. Osmotic fragility values of donor's blood after incubation with PS-AChR-liposomes. (A) Histograms obtained after the incubation with different concentrations of PS-AChR-liposomes **(B)** AUC (Area Under the Curve) calculated from the histograms of every donor. Violin plots represent control group (dark green), 5 minutes timepoint (dark pink), 30 minutes timepoint (medium pink) and 120 minutes timepoint (light pink). No significant differences were found when comparing among different conditions (Kruskal-Wallis test with Dunn's multiple comparisons test).

3. PS-AChR-liposomes do not induce secretion of cytokines associated with CRS

A critical aspect of the safety profile of novel immunotherapies involves assessing their potential impact on systemic inflammation. Given that PS-liposome therapy is intended for IV administration, we performed an evaluation of its effects on the bloodstream. This assessment was essential to determine whether PS-liposome administration induces an excessive release of proinflammatory cytokines associated with CRS, which is a potentially life-threatening condition characterized by elevated

circulating cytokine levels, acute systemic inflammatory symptoms, and secondary organ dysfunction.

To evaluate the potential of PS-liposomes to trigger CRS, three experimental conditions used in the previous regulatory assays were incubated with human donor blood for 4, 24, and 48 hours. Detailed information about the blood donors and experimental setup is presented in **Table 20**. LPS was used as a positive control, as it is known to induce CRS and is widely utilized in the LPS-Induced Cytokine Release Model [406]. PBS was used as a negative control.

Table 20. Data from control subjects included in the CRS test

Parameter	Control subjects
N	5
Gender	3/5 (60%) Female 2/5 (40%) Male
Age (years)	25.67 ± 1.75

Data presented as mean±SD.

The release of proinflammatory cytokines was analysed, encompassing IL-1 β , IL-6, IL-8, IFN- γ , IL-17A, and TNF- α . IL-1 β , IL-6, and IL-8 which showed a significant increase exclusively in the LPS condition, while no significant increment was observed in either the PS-liposome-treated samples, regardless of the concentration, or the PBS control (**Figure 38**). IFN- γ and IL-17A exhibited no significant differences among conditions at any time point; however, a natural time-dependent increase in the release of these cytokines was perceptible, reaching statistical significance at 24 and 48 hours in comparison to 4h timepoint. Conversely, TNF- α levels increased dramatically in the LPS condition as early as 4 hours post-incubation, with a progressive rise over the subsequent 24 and 48 hours. Notably, at the 48-hour time point, a slight increase in TNF- α was observed in both the PS-liposome-treated samples and the PBS control, likely reflecting the natural cytokine release associated with *ex vivo* blood incubation.

RESULTS

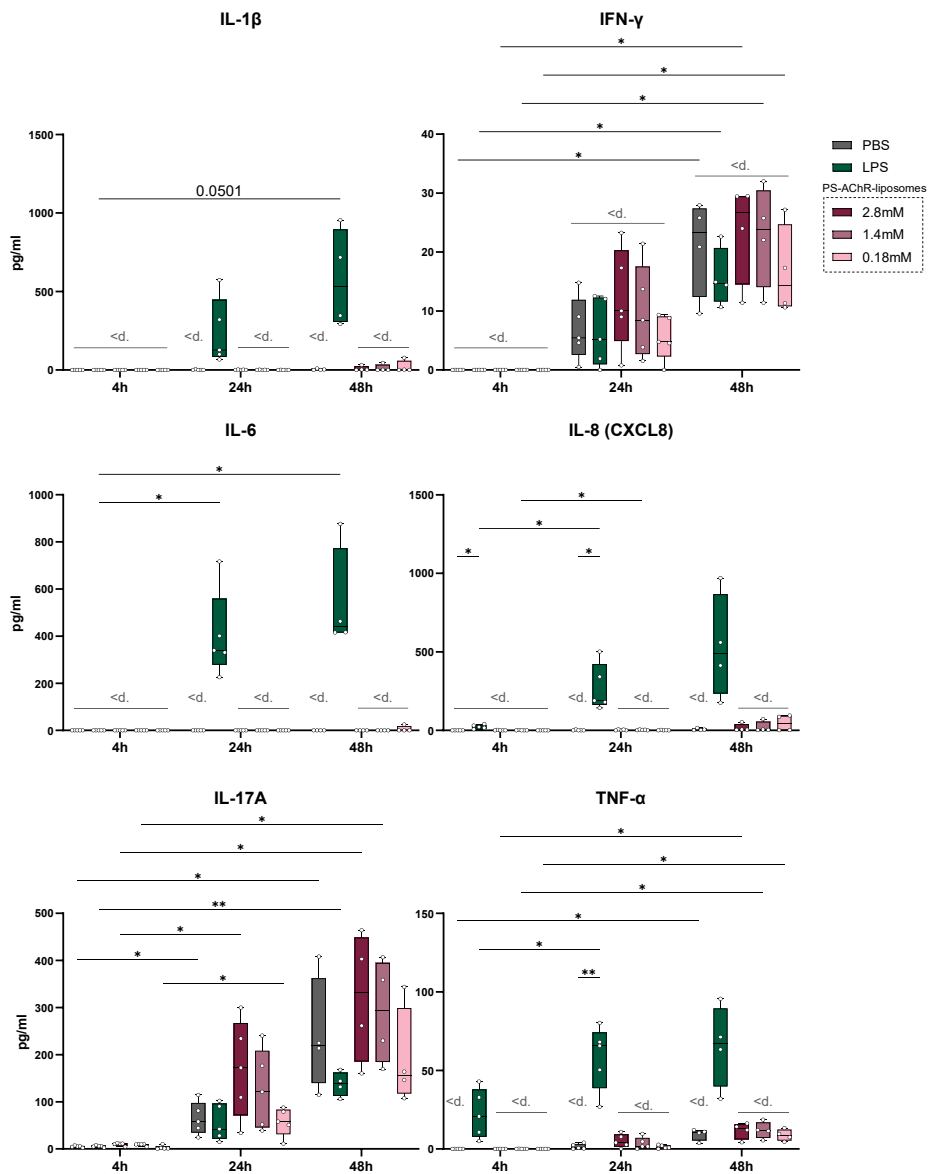


Figure 38. Functional effect of the PS-AChR-liposomes in the release of pro-inflammatory cytokines. Levels of IL-1 β , IFN- γ , IL-6, IL8, IL-17A and TNF- α secreted by bloodstream immune cells obtained from control subjects (n $\geq$ 4). Boxes represent negative control group –conditions cultured with PBS– (grey), positive control group –conditions cultured with LPS– (green), conditions cultured with 2.8mM of PS-AChR-liposomes (dark pink), conditions cultured with 1.4mM of PS-AChR-liposomes (medium pink) and conditions cultured with 0.18mM of PS-AChR-liposomes (light pink); when a condition has data calculated under the standard curve a (<d.) symbol is indicated. Data is presented as a mean $\pm$ SD. Significant differences were found when comparing among different conditions (*p<0.05, **p<0.01, Mixed-effects analysis with Dunnett's multiple comparisons test).

Regarding the release of cytokines associated with immune regulation and hematopoietic growth, both G-CSF and GM-CSF levels significantly increased after 24 and 48 hours of incubation under LPS-stimulated conditions, as expected (**Figure 39**). Neither PBS nor PS-liposome-treated samples exhibited statistically significant differences when compared to negative controls. Notably, the lowest concentration of PS-AChR-liposomes after 48h presented a single outlier, however, this did not influence the overall statistical analysis. Similarly, IL-2, IL-4, and IL-5 followed a comparable trend, with a progressive increase in cytokine levels observed in the positive control condition starting at 4 hours, reaching statistical significance at both 24- and 48-hours post-incubation. In contrast, PS-AChR-liposome conditions presented only a slight, non-significant elevation in these interleukins, suggesting a baseline rise in cytokine levels potentially attributable to *ex vivo* culture stress rather than a specific immunostimulatory effect of the liposomes. IL-12p70 secretion was markedly induced exclusively in the LPS-treated samples, in agreement with existing literature reporting its upregulation in LPS-stimulated M ϕ and DCs [407]. All other experimental conditions, including those involving PS-liposomes, did not result in significant alterations in IL-12p70 levels throughout the time course.

Concerning the chemokines and IL-18 (**Figure 40**), IP-10 was significantly upregulated exclusively in the LPS-stimulated condition after 48 hours of incubation, as compared to the PBS negative control. In contrast, MCP-1 levels did not exhibit significant differences across any of the tested conditions, although considerable inter-donor variability was observed. Notably, the PS-liposome-treated conditions did not surpass the baseline levels seen in the negative control, suggesting a lack of chemotactic activation. Both MIP-1 α and MIP-1 β followed a similar pattern, with secretion significantly increased only in the LPS positive control group, showing a time-dependent escalation throughout the incubation timepoints. Finally, IL-18 secretion, however, displayed a generalized increase across all conditions after 24 and 48 hours of *ex vivo* incubation, likely reflecting a stress response associated with prolonged blood culture outside physiological conditions.

RESULTS

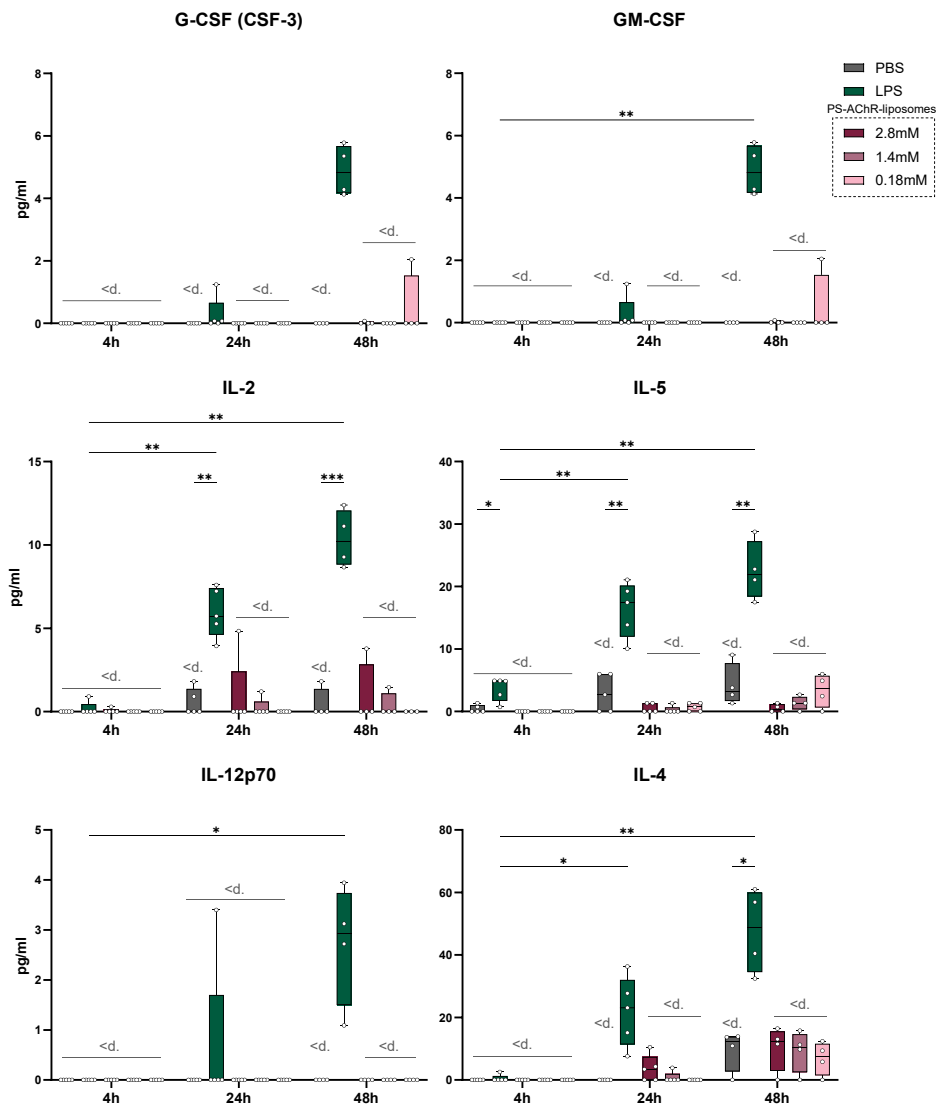


Figure 39. Functional effect of the PS-AChR-liposomes in the release of cytokines involved in immune regulation and growth. Levels of G-CSF (CSF-3), GM-CSF, IL-2, IL-5, IL-12p70 and IL-4 secreted by bloodstream immune cells obtained from control subjects (n≥4). Boxes represent negative control group –conditions cultured with PBS– (grey), positive control group –conditions cultured with LPS– (green), conditions cultured with 2.8mM of PS-AChR-liposomes (dark pink), conditions cultured with 1.4mM of PS-AChR-liposomes (medium pink) and conditions cultured with 0.18mM of PS-AChR-liposomes (light pink); when a condition has data calculated under the standard curve a (<d.) symbol is indicated. Data is presented as a mean±SD. Significant differences were found when comparing among different conditions (*p<0.05, **p<0.01, Mixed-effects analysis with Dunnett's multiple comparisons test).

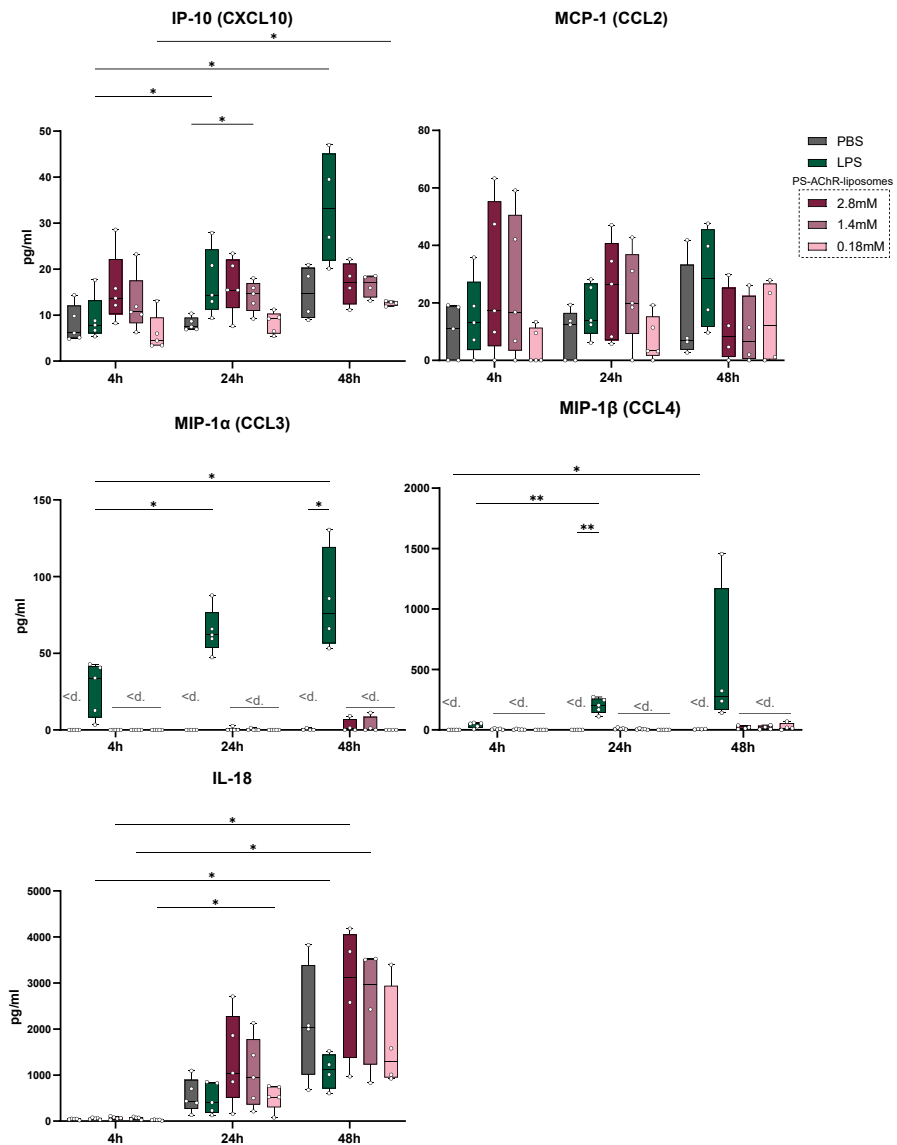


Figure 40. Functional effect of the PS-AChR-liposomes in the release chemokines and viral infection related cytokines. Levels of IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4) and IL-18 secreted by bloodstream immune cells obtained from control subjects (n≥4). Boxes represent negative control group –conditions cultured with PBS– (grey), positive control group –conditions cultured with LPS– (green), conditions cultured with 2.8mM of PS-AChR-liposomes (dark pink), conditions cultured with 1.4mM of PS-AChR-liposomes (medium pink) and conditions cultured with 0.18mM of PS-AChR-liposomes (light pink); when a condition has data calculated under the standard curve a (<d.) symbol is indicated. Data is presented as a mean ± SD. Significant differences were found when comparing among different conditions (*p<0.05, **p<0.01, Mixed-effects analysis with Dunnett's multiple comparisons test).

4. PS-liposomes trigger a dose-dependent expression of a tolerogenic marker candidate for the potency assay

The term *potency* refers to the specific ability or capacity of a product to achieve a desired outcome, as demonstrated by appropriate laboratory tests or well-controlled clinical data gathered through the intended use of the product. The potency assay should reflect the mechanism of action of the drug to be developed. Among the available options, cell culture-based biological assays are the most commonly used, despite exhibiting higher variability than biochemical assays or ligand-receptor binding assays, they allow measurement of the biochemical or physiological response at the cellular level and therefore making them more representative of the biological response of an organism to the product.

Interestingly, after the incubation of DCs from MG and RA patients with varying concentrations of PS-loaded liposomes, a notable modulation of TOLX surface expression was observed (**Figure 41**). While this modulation was also detectable under immature conditions, it became statistically significant in the matured DCs, likely due to their inherently higher baseline expression of TOLX. The dose-dependent effect was particularly evident in the mature compartment, with higher concentrations of PS-loaded-liposomes correlating with increased TOLX surface expression. This concentration-dependent regulation of this marker highlights a reproducible and quantifiable response, paving the way for the development of a potency assay based on TOLX expression, which could be a useful tool for the future regulatory applications.

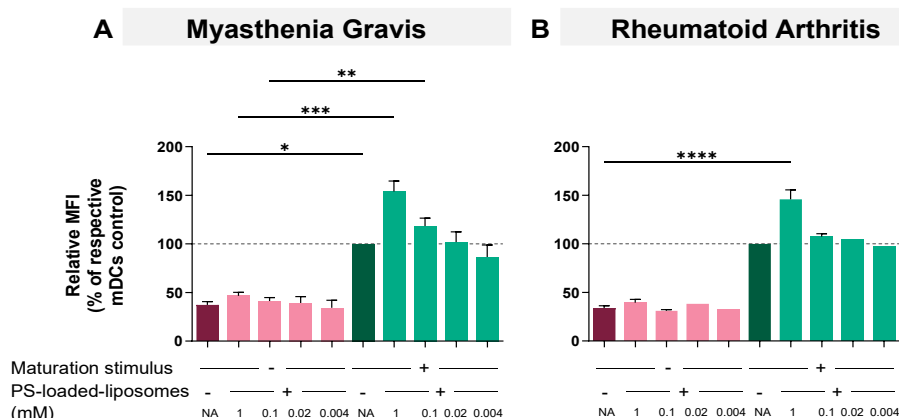


Figure 41. TOLX dose-response expression levels in DCs from MG and RA patients after PS-loaded-liposomes phagocytosis. (A) DCs obtained from MG (n= 4-11) and (B) RA patients (n= 1-9). Bars represent iDCs (dark pink), iDCs after PS-loaded-liposome phagocytosis (light pink), mDCs (dark green) or mDCs after PS-loaded-liposome phagocytosis (light green). Data is presented as a mean±SD. Significant differences were found when comparing among different conditions (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Two-way ANOVA with Tukey's multiple comparison test).

Cells are the most critical component in the development of a cell-based potency assay as the selected cell type must support a quick test reproducible, and biologically relevant readout. While primary cells often provide physiologically meaningful data, they are subject to significant donor-to-donor variability and limited in vitro lifespan, making them less suitable for standardized assays. Consequently, immortalized cell lines are generally preferred for potency assays due to their stability and scalability. Building on prior observations that PS-liposomes interact with B cells (**see section III**), we started the dose-response tests with the commercially sourced TANOUE B cell line. Furthermore, recent studies have reported that some tumoral cell lines have the ability to engulf AC, prompting us to check the ability of TANOUE cells to uptake NBD-labelled-PS-liposomes [408,409]. Remarkably, these cells demonstrated higher uptake levels than primary B cells isolated from PBMCs (**Supplementary Figure 28A**). Importantly, TANOUE cells also exhibited a dose-dependent increase in TOLX surface expression following 24 hours of incubation with increasing concentrations of PS-liposomes, mirroring the response observed in DCs from patients. This effect was successfully modelled using the Padé equation, yielding a regression line with a strong correlation coefficient ($R^2 = 0.928$) (**Supplementary Figure 28B-C**). However, it is worth noting that with successive culture passages, the reproducibility of results declined, and variability increased,

thereby limiting the long-term utility of this cell line for potency assay standardization.

Given the limitations of the B cell line, an alternative strategy was pursued by developing the potency assay using human PBMCs. One of the key advantages of using PBMCs is their availability from commercial biobanks, which ensures a consistent and traceable source of qualified cells screened for contaminants and suitable for use in regulated assays. To establish the assay parameters, PBMCs from healthy donors recruited at the Hospital Germans Trias i Pujol (Badalona, Spain) were initially used (**Table 19 and 20**). An extensive concentration gradient of PS-AChR-liposomes was applied in this setting to accurately determine the IC_{50} value, which serves as a critical pharmacological parameter reflecting the concentration required to achieve a 50% modulation of the biological response. Key assay variables, including the number of replicates, liposome volume per well, and cell concentration, were carefully optimized to enhance reproducibility and precision. The surface expression of TOLX on B cells within the PBMC fraction demonstrated a well-defined and reproducible dose-response relationship. Specifically, higher concentrations of PS-AChR-liposomes induced a progressive increase in MFI, with the highest dose (2 mM) resulting in a $217.62 \pm 38.33\%$ of increased TOLX expression relative to the unstimulated condition (**Figures 42A and B**). When normalized and interpolated through regression analysis, the dose-response curve showed a strong correlation coefficient ($R^2 = 0.800$) and a half-maximal inhibitory concentration (IC_{50}) of 0.84mM, confirming the robustness and predictive value of this approach for future potency assay development (**Figure 42C**).

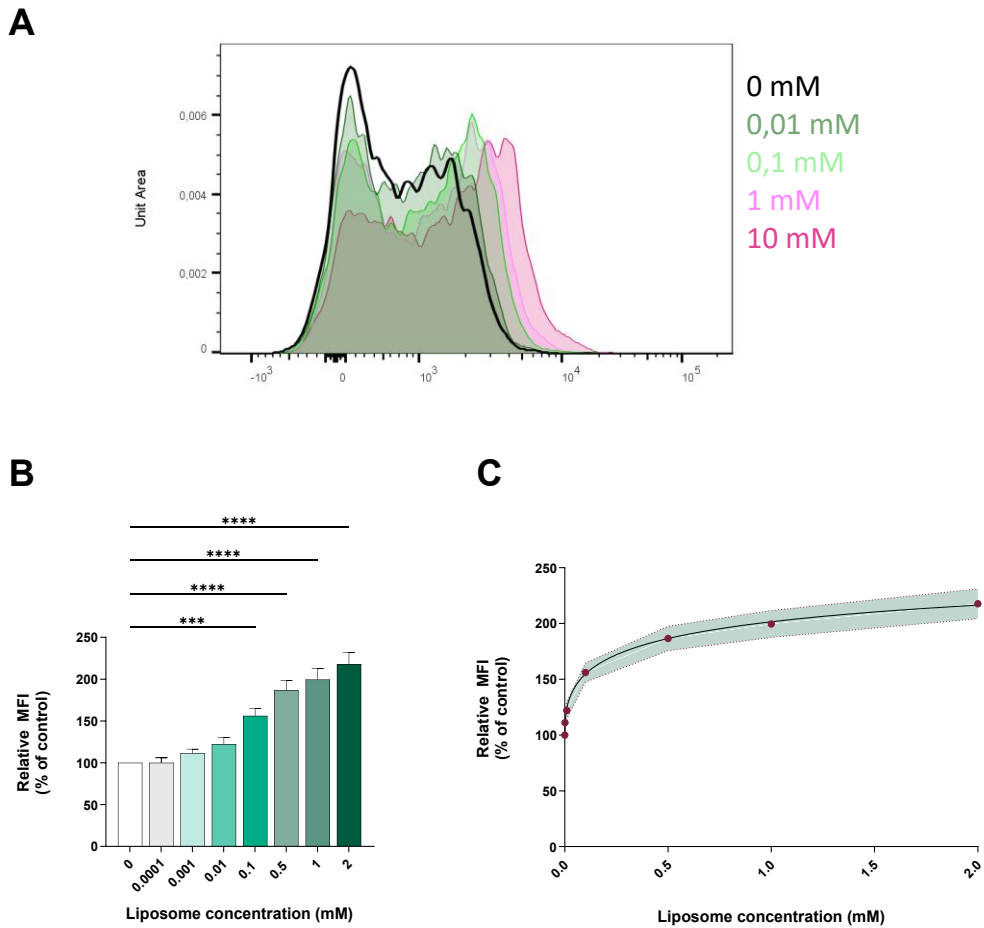
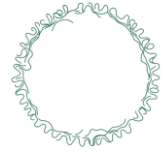
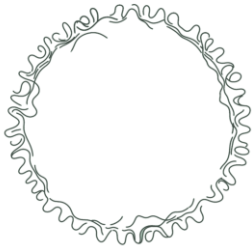
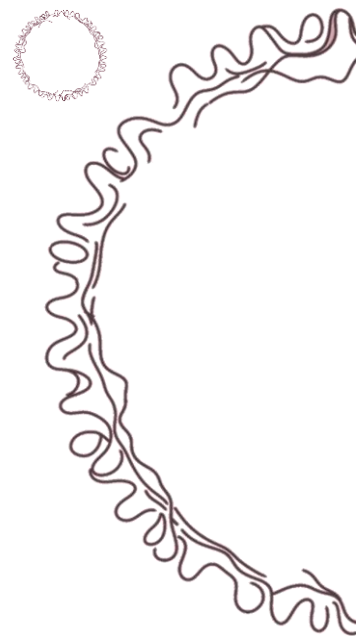


Figure 42. PS-AChR-liposome dose response expression of TOLX on B cells from control subjects. (A) Histogram of a representative TOLX flow cytometry positivity in B cells after incubation of a range of PS-AChR-liposome concentration. **(B)** Relative MFI of TOLX in B cells after interaction with a gradient of PS-AChR-liposome concentrations ($n=3-7$) **(C)** and the correspondent regression curve performed. Bars represent with a gradual scale from white to dark green the increasing concentration of PS-AChR-liposomes. Data is presented as a mean $\pm$ SD. Significant differences were found when comparing among different conditions (** $p<0.001$, **** $p<0.0001$, Ordinary one-way ANOVA with Holm-Šidák's multiple comparisons test).



5



DISCUSSION

Autoimmune diseases arise from a breakdown in immunological tolerance, leading to aberrant immune responses against self-antigens. Over the past decades, the incidence of autoimmune diseases has steadily increased, now affecting approximately 10% of the global population [47–49]. This rise poses a significant economic burden on healthcare systems due to the absence of a definitive treatment capable of halting the autoimmune attack. Current therapeutic approaches primarily rely on replacement therapies, symptomatic treatments, or broad-spectrum immunosuppressive drugs. While these first-line treatments have proven effective in alleviating the severity of the disease and improving patients' quality of life, they do not address the underlying immune dysregulation and therefore do not provide a cure for the disease. The advent of biologic therapies has substantially enhanced treatment outcomes by reducing adverse effects and offering more targeted immunomodulation. However, these interventions fail to restore immune tolerance, limiting their long-term efficacy. Recently, new therapeutic strategies have emerged to reverse autoimmune responses by re-educating the immune system for therapeutic curative aims. Although numerous experimental approaches are under investigation, none have yet reached routine clinical application. These innovative immunotherapies seek to suppress antigen-specific pathogenic immune cells while preserving protective immunity, thereby establishing durable and antigen-specific tolerance. The primary principle underlying these strategies involves the administration of the autoantigen responsible for triggering the autoimmune response, enabling the highly selective modulation of immune activity while maintaining immune surveillance and minimizing side effects. Ideally, these therapies should also induce immunological memory to ensure sustained suppression of the autoimmune attack. Under optimal conditions, one of these approaches may first progress through clinical trials and ultimately achieve clinical implementation. However, even if only a modest therapeutic efficacy is demonstrated, antigen-specific strategies will contribute valuable insights into the pathogenesis of autoimmune diseases and pave the way for refining immunomodulatory interventions, further advancing the field of immune system re-education. In the present doctoral thesis, we focused on the preclinical assessment of PS-loaded-liposomes to gain an in-depth understanding of how the immunotherapy works to design the best approach and to ensure efficacy and safety in the future clinical trial.

PS-liposomes mimicking apoptotic bodies as a potential immunotherapy for autoimmunity

In addition to central and peripheral tolerance mechanisms, which primarily function to prevent autoreactivity, the immune system has additional physiological processes that contribute to immune tolerance and protect against self-destruction. One such mechanism is efferocytosis, a process in which APCs efficiently engulf AC [410]. During this process, immune homeostasis is preserved through the rapid and efficient removal of AC, which occurs in a non-inflammatory environment and also promotes tolerogenic signalling by APCs, thereby preventing immune activation against self-antigens. When apoptotic cells are not efficiently cleared, they progress to secondary necrosis, leading to the release of intracellular contents along with danger-associated molecular patterns (DAMPs) and pro-inflammatory signals. Notably, defects in apoptotic cell clearance have been implicated in the pathogenesis of several autoimmune diseases, underscoring the critical role of efferocytosis in maintaining immune homeostasis [310–316].

An innovative therapeutic strategy has been developed leveraging the immunomodulatory properties of apoptotic bodies to reverse pathological immune activation in T1D [411]. As a proof of concept, apoptotic β -cells were co-cultured with murine DCs, resulting in the induction of a tolerogenic phenotype. Subsequent reintroduction of these cells into NOD mice led to a decreased incidence of T1D [383]. Furthermore, in the context of antigen-specific cellular immunotherapy, the reduction of inflammatory cytokines and the release of PGE₂ were identified as key factors responsible for the immunomodulatory effects observed following AC uptake [384]. However, the translation of apoptotic β -cell approach to human therapy has been hindered by the challenges associated with the collection, preservation, and standardization of autologous human β -cells, which remains practically unfeasible. To overcome this limitation, a promising solution emerged in the form of nanoparticles designed to mimic apoptotic bodies. This strategy takes advantage of the **“eat-me” signal provided by PS**, exposed in the outer leaflet of the plasma membrane of the AC. The PS-liposome strategy relies on the apoptotic mimicry, which, as mentioned previously, facilitates the clearance of apoptotic cells in a homeostatic, immunologically silent manner, thereby preventing inflammation and promoting tolerance to the autoantigens they contain. Notably, the recognition of externalized PS by APCs triggers conserved downstream signalling pathways that induce homeostatic programs leading to non-inflammatory and non-immunogenic outcomes, thereby maintaining immune tolerance [357]. Interestingly, certain

pathogens and tumour cells have evolved mechanisms to exploit apoptotic mimicry to evade immune surveillance. For example, both enveloped and non-enveloped viruses use the termed viral apoptotic mimicry to facilitate cell entry and avoid immune detection [412,413]. Similarly, some bacteria appear to exploit efferocytosis and PS externalization for similar purposes. Tumour cells also hijack the non-immunogenic properties of apoptotic cells by promoting local immune suppression through PS externalization or the release of PS-rich tumour exosomes [414,415]. Indeed, both pathogens and cancer cells using apoptotic cell mimicry to evade immune responses underscores the significance of the PS-mediated efferocytosis as a tolerance-inducing process and highlights its potential utility in immunotherapy.

For this reason, PS-liposomes have been engineered with specific **physicochemical properties** and components to mimic seamlessly apoptotic bodies. The PS-liposomes were designed to exhibit a range of diameters while maintaining an average particle size of 500 nm, ensuring efficient phagocytosis by APCs rather than passive uptake mechanisms, which are predominantly utilized by smaller particles [416]. In terms of composition, PS-liposomes were formulated with CH and PC for structural purposes, with CH playing a crucial role in maintaining membrane fluidity and integrity [417]. The third phospholipid, PS, was incorporated as the key component responsible for mediating PS recognition and efferocytosis. Additionally, PS contributes a negative surface charge, which has been associated with anti-inflammatory properties [385,418,419]. This results in a negative ζ -potential, which confers less toxicity than positively charged NPs [420].

Structurally, the hydrophilic head groups of the phospholipids are oriented towards the aqueous environment (both externally and internally), while the hydrophobic fatty acyl chains are positioned within the bilayer. This configuration favours the formation of a vesicular structure capable of encapsulating peptides. Regarding **encapsulation efficiency**, PS-loaded liposomes generally exhibited high peptide-loading capacity, with yields exceeding 70%. However, certain peptides demonstrated lower encapsulation efficiency, likely due to variations in amino acid composition, solubility, or charge. These findings, in conjunction with the demonstrated immunomodulatory properties of PS, highlight the therapeutic potential of PS-loaded liposomes. Not only do they serve as effective delivery vehicles capable of encapsulating various peptides, thereby enabling adaptation to different antigen-specific autoimmune diseases, but they also facilitate targeted delivery to DCs, M ϕ , or other phagocytic cells expressing PS receptors. Furthermore, their straightforward production process and established stability reinforce their suitability as an ideal platform for the development of immunoregulatory therapies.

Biodistribution and clearance of PS-liposomes by phagocytes in mice models

The evaluation of a new drug's **biodistribution** is a crucial step in both regulatory approval and therapeutic development, as it provides key insights into its pharmacokinetics, efficacy, and potential off-target effects. The route of administration plays a significant role in determining the uptake, targeting efficiency, and overall immunomodulatory properties of PS-liposomes. Therefore, selecting the most appropriate administration route is essential to ensure both safety and therapeutic efficacy. Several liposomal-based drug delivery systems have already been approved by the U.S. Food and Drug Administration (FDA) for clinical use in disease treatment [421]. Moreover, they are commonly used for diagnosis and therapeutical purposes using different administration routes including intraocular, oral, intranasal, transdermal or parenteral [422–426]. Until now, PS-liposomes have been administered IP into murine models. However, its difficult translation to humans and seeing that other ASIT therapies in development opt for other routes of administration, in **SECTION I**, we evaluated four distinct administration routes – IP, ID, IV, and IN– in order to characterize the spatial and temporal distribution of PS-liposomes following administration [427].

Notably, the IN administration of empty PS-AF750-liposomes in prediabetic female NOD mice showed targeted distribution particularly to lungs, as expected [428,429]. Considerable fluorescence was also detected in the stomach, likely due to an accidental ingestion, which could introduce variability in biodistribution. This route, in general, showed a rapid clearance of deposited PS-liposomes but only a minimal migration to lung-draining LN, suggesting that PS-liposomes mostly did not reach the gas-exchange region where alveolar M ϕ or professional phagocytes could facilitate lymphatic transport [430,431]. In addition, published clinical trials using this administration route, attempting to nasal mucosal tolerance to tackle T1D, showed low efficacy and very heterogeneous results, partly due to the nature of the delivery strategy, which could be related with the accelerated exposure to enzymatic degradation [432]. ID delivery, characterised by administration of the NP directly through the skin layers to skin-resident enriched APCs, this time using PS-liposomes, showed the lowest fluorescence levels among all routes tested, with the signal initially gathering around the injection site. Moreover, fluorescence persisted in certain organs even after 24 hours, possibly due to the limited absorption of larger particles. Unlike smaller colloidal systems, which rapidly enter capillaries, larger particles are retained at the injection site and slowly drain into the lymphatic system,

highlighting the importance of careful site selection to optimize absorption efficiency [433]. IP administration initially presented high fluorescence rates in organs adjacent to the injection site, consistent with the direct delivery of labelled nanoparticles into the peritoneal cavity, also seen in other published studies using this route [434]. Notably, while the IP route capacity to reach secondary lymphoid organs, residual fluorescence detected in certain tissues after 24 hours suggests a slower systemic distribution to distant target organs. This route has already been approved in specific clinical contexts for the administration, alone or combined, of NP-based drug delivery systems, particularly in targeting peritoneally localized malignancies such as ovarian, breast and pancreatic cancers [435,436]. Despite its widespread use in rodent models due to ease of administration, the limited translational potential of IP delivery to clinical settings—owing to high variability in bioavailability and potential for misinjection— renders it less suitable for therapeutic applications in humans.

In contrast, **the IV route** appears to be a more promising option in terms of clinical translatability. Although some ASIT therapies have failed using the IV route of administration [437,438], it is increasingly common to see clinical trials using this route of administration [257,283]. Our biodistribution studies revealed a consistent distribution pattern regardless the mice strain or sex. Regarding kinetic profiles, IV administration confirmed a rapid systemic distribution of the PS-liposomes in nearly all examined organs within just 15 minutes post-injection. As seen in other NP-formulated therapies biodistribution, this route presented enhanced delivery to the spleen and liver, organs that play crucial roles in immune tolerance due to their specialized immune environments and functions [439,440]. Additionally, IV administration of PS-liposomes reached remarkably secondary lymph nodes. Interestingly, the bladder was the only organ presenting the highest fluorescence levels at 6h post-injection, suggesting probably the excretion of the smallest PS-liposomes, not captured by the RES or MPS, through the renal pathway due to the pore size limit of glomerular filtration in the kidneys [441]. Noteworthy, after 48h post-administration PS-liposome signal was nearly undetectable in all examined organs, indicating an absence of long-term bioaccumulation and reducing the risk of toxicity.

ASIT therapies must reach innate and adaptive immune cells in order to effectively promote immune tolerance. Having established that IP, ID, and IV administration routes enable the delivery of PS-liposomes to immunologically relevant organs, including secondary lymph nodes and the liver, the evaluation of interacting immune cell subsets with NBD-labelled PS-liposomes *in vivo* within these organs was performed. At 6h timepoint, in general, all resident APCs within each organ displayed

positive interaction, as expected, with no significant differences observed between empty or loaded NBD-liposomes, suggesting a similar biodistribution and uptake pattern. The ID administration showed low percentages of capture; however, APCs such as cDCs and pDCs, although with considerably lower efficiency than professional phagocytes, exhibited NBD positivity in the spleen, MDLN, and PLN, demonstrating their ability to capture PS-liposomes in the dermis and migrate to secondary lymphoid organs. In contrast, IP administration resulted in the highest percentages of PS-liposome uptake, likely due to its slower biodistribution kinetics, as previously observed. This phenomenon may be explained by the initial interaction of PS-liposomes with resident immune cells in the peritoneal cavity, followed by migration through peritoneal draining lymphatics to secondary lymph nodes, where they present antigens and influence adaptive immune responses. A particularly interesting finding was the interaction of PS-liposomes with B cells, especially in the MDLN, likely attributable to the presence of B1 cells, which have established phagocytic functions in murine models, particularly B1a and B1b subsets from the peritoneal cavity, capable of antigen presentation via MHC class II to T cells [442,443]. In contrast, IV administration led to an immediate systemic distribution reaching the spleen and LN via circulation. After 6h, the percentage of capture in those organs was low, likely due to faster clearance and redistribution. However, it is noteworthy that phagocytes such as cDCs in the spleen and PLN exhibited high levels of capture, suggesting either increased bioavailability of PS-liposomes in these organs or active migration of cDCs to these lymphoid tissues, where they may present antigens and regulate T cell activation.

To further decipher the dynamics of IV administration of PS-liposomes and their clearance by immune cells in the two major organs supplied by the bloodstream, a series of time-course experiments were conducted in both NOD and C57BL/6 mice using NBD-labelled PS-Ins-liposomes and PS-AChR-liposomes, respectively. Notably, both formulations presented comparable interactions and uptake by immune cell subsets in the spleen and liver, regardless of the encapsulated peptide. This finding suggests that the PS composition of the liposomes is the key determinant of their cellular interactions. Upon IV administration, PS-liposomes are immediately introduced into circulation where they interact with various blood components, including plasma proteins and immune cells before being distributed to different organs. During the early phase of circulation, PS-liposomes can interact with complement factors and opsonins such as immunoglobulins and pentraxins, which may facilitate their recognition by phagocytic cells [444,445]. Although not for the first time, here we reported that among the PBMCs, monocytes despite being in

circulation were able to internalize NPs intravenously administered [446]. Neutrophils, despite constituting a smaller proportion of circulating leukocytes in mice compared to humans, represent 20-30% of blood cells in mice and exhibit significant phagocytic potential [447]. Here, although the percentage of neutrophils capturing PS-liposomes was relatively low, the absolute number of these cells engaging in phagocytosis was substantial. Additionally, recent evidence has expanded the role of neutrophils beyond innate immunity, highlighting their ability to modulate adaptive immune responses by suppressing T cell proliferation and activity and the ability to interact with immunomodulatory nanoparticles as well [448,449]. Furthermore, other phagocytic cells, including different subsets of DCs and M ϕ , also showed positivity percentages of PS-liposomes uptake, despite their lower frequency in circulation. Despite the consistency of the results, certain limitations should be considered, especially in short timepoints. APCs may continue phagocytosis of PS-liposomes after sample collection. While the use of EDTA tubes chelates calcium and limits monocyte phagocytic activity, and fixation halts biological processes, additional pharmacological inhibitors could be employed to confirm *in vivo* phagocytosis. Agents such as Cytochalasin D or Dynasore that inhibit actin polymerization or block endocytic pathways are effective options [450,451]. More specifically, PS-dependent phagocytosis could be specifically inhibited using Annexin V or other PS-binding proteins.

As circulation continues, PS-liposomes are transported via the caudal vein toward the heart, where they enter the right atrium, pass into the right ventricle, and are pumped through the pulmonary arteries to the lungs. After traversing the pulmonary circulation and interacting with pulmonary APCs such as M ϕ , oxygenated blood carrying PS-liposomes returns to the left atrium and is subsequently distributed throughout the body via the aorta. One of the first major organs encountered is the **liver**, which receives approximately 25–27% of the cardiac output and is characterized by an extensive network of sinusoidal capillaries [452]. The liver is known as a central hub for immune tolerance, owing to its unique microenvironment, distinct immune cell composition, and immunoregulatory mechanisms. It is continuously exposed to high concentrations of microbial and food-derived antigens from the gastrointestinal tract. This persistent antigenic exposure fosters immune tolerance, preventing hypersensitivity reactions to harmless antigens. Moreover, the liver's tolerogenic properties are further exemplified by its ability to accept allografts despite MHC mismatches, as demonstrated in transplantation studies [440]. The literature suggests that between 30% and 99% of administered nanoparticles are sequestered by the liver, a process influenced by

nanoparticle properties such as dose, shape, and size, typically ranging from 50 to 500 nm [453,454]. The primary mechanism of hepatic NP clearance involves the RES or MPS, which is highly active in liver sinusoids. Kupffer cells, the liver's resident M ϕ , are the predominant phagocytic population in this organ, residing within the luminal side of sinusoidal capillaries and maintaining direct contact with circulating nanoparticles [455]. In this study, Kupffer cells exhibited almost 100% positivity for NBD-labelled PS-liposomes within the first six hours post-administration, highlighting their rapid and efficient uptake. Additionally, moM ϕ and monocytes also demonstrated high levels of phagocytosis within the first 15 minutes. The ability of these subsets to engulf PS-liposomes suggests that their degradation occurs in an immunologically silent manner, potentially promoting a tolerogenic immune response through the release of anti-inflammatory cytokines and antigen-specific modulation of T cell activity [456,457]. Moreover, recent publications abrogate for the antigen-specific tolerance induction capacity of Kupffer cells since the adoptive transfer of these cells, which uptake NPs coupled with an antigen, significantly mitigated the clinical score of EAE in mice [458]. We also underscored the LSECs role in the PS-liposome clearance. Due to their abundance and strategic positioning within liver sinusoids, these highly fenestrated cells, with pores measuring approximately 100-140 nm, are particularly permeable to small nanoparticles. In addition, LSECs express PS receptors, facilitating active uptake of PS-liposomes within the first hour post-administration [459]. LSECs possess the capacity to cross-present antigens to CD8⁺ T cells and induce a negative feedback inhibition via interferon signalling, demonstrating their critical role in immune regulation [460–462]. Furthermore, other ASIT therapies targeting specifically LSECs have achieved an antigen-specific CD4⁺ FoxP3⁺ Tregs expansion, highlighting its role in tolerance induction [288]. All DCs subsets showed a similar pattern of phagocytosis, reaching peak uptake levels approximately 1-hour post-exposure, suggesting their role as a secondary barrier in PS-liposome clearance. Despite their lower phagocytic capacity compared to Kupffer cells, DCs should not be underestimated due to their ability to migrate to draining lymph nodes and present antigens to naïve T cells, thereby influencing adaptive immune responses. Finally, although at lower frequencies, PS-liposomes also interacted with B and T cells within the first hour following administration, further underscoring their broad immunological impact.

Chronologically, the next organ that PS-liposomes reach after circulation through the liver is the **spleen**, a specialized organ responsible for filtering foreign materials and aged RBC from systemic circulation, representing a key organ in the induction of immunological tolerance. Upon arrival, larger PS-liposomes become trapped, and red

pulp M ϕ , specialised in the aged RBC clearance, display high percentages of NBD fluorescent PS-liposomes uptake. cDC2 subset, typically located in the red pulp and marginal zone and which play a critical role in antigen processing and presentation to CD4⁺ T cells via MHC class II molecules, also showed NBD positivity, highlighting their involvement in PS-liposome uptake [463]. Notably, cDC1s, in the white pulp and specialized in cross-presenting antigens to CD8⁺ T cells, display even higher levels of PS-liposome uptake, suggesting a strong potential for antigen presentation and immune modulation [464,465]. Additionally, monocytes and MoM ϕ , participated in the PS-liposome uptake further supporting the role of phagocytes in processing these particles. The detection of NBD fluorescence in white pulp and marginal zone M ϕ suggests that PS-liposomes not only interact with antigen-presenting cells in the red pulp but also reach compartments where adaptive immune responses are initiated. These findings indicate that M ϕ and DCs may facilitate antigen presentation to both T and B cells, thereby potentially influencing adaptive immune responses. The spleen immune tolerance induction properties are also exploited by many other ASIT therapies. Several studies with experimental autoimmune models driven by Th1 and/or Th17 lymphocytes have proven, by IV administration of either synergic splenocytes or erythrocytes coupled or loaded with antigens, to induce potent antigen-specific tolerance *in vivo*. An effect that relies on the clearance of apoptotic cells and dying erythrocytes by the splenic phagocytes, IL-10 production and expansion of antigen-specific Tregs [439].

Over the course of 6-hours, PS-liposomes continue to be processed by cells of the MPS. However, by 24 hours—and particularly at 48 hours—the fluorescence signal in all immune cell subsets is substantially reduced to undetectable levels. This decline suggests that PS-liposomes undergo further lysosomal degradation, leading to efficient clearance without bioaccumulation in any organ.

Overall, IV administration of PS-liposomes resulted in their rapid systemic distribution, with initial interactions occurring in the bloodstream, followed by predominant uptake by the liver and spleen via the RES and MPS. Our data suggests that the therapeutic effect observed upon administration of PS-liposomes relies, at least, partially, on the non-inflammatory antigen delivery to the liver and spleen. Moreover, confirms that PS-loaded-liposomes is an excellent antigen delivery platform, being cleared as AC by professional phagocytes which potentially facilitate antigen presentation and immune modulation. Altogether, our findings highlight intravenous administration as a promising strategy for immune modulation by PS-loaded-liposomes. Moreover, other NP-formulated therapies have already been approved for IV administration [466]. However, some limitations of extrapolating

administration route data from murine models to humans should be taken into account, as the physiological, anatomical, and immunological cell distribution can differ [467,468]. Moreover, translational challenges must be emphasised when selecting a clinically appropriate administration route. Factors such as ease of administration, patient compliance, invasiveness, and compatibility with repeated dosing must all be considered in order to achieve the most efficient and strategic approach possible.

Antigen-specific immunomodulation of loaded PS-liposomes

Inducing antigen-specific immune tolerance is fundamental for developing effective therapies for autoimmune diseases, as it enables selective suppression of pathogenic immune responses without compromising overall immune function. In **SECTION II**, we demonstrated that PS-loaded-liposomes encapsulating autoantigens and predominantly targeting APCs, aim to relay a tolerogenic signal to T lymphocytes in an **antigen-specific** fashion. The successful reduction of T1D incidence in NOD mice following treatment with PS-Ins-liposomes highlighted the potential of antigen-specific modulation. Notably, previous studies using MHC class II tetramer analysis have shown that PS-2.5mi-liposomes induce antigen-specific expansion of CD4⁺ T cells, including both FoxP3⁺ and FoxP3⁻ subsets, when administered to prediabetic NOD mice [411]. This expansion was further associated with an increase in the Tr1 cell markers LAG3 and CD49b, suggesting that PS-loaded liposomes promote the differentiation of both conventional and non-conventional regulatory T cells, which play a key role in immune tolerance through the release of immunomodulatory cytokines such as IL-10, however the negative expression of FoxP3 was not verified [389,469]. To further discern this mechanism, in this study we used the NODBDC-2.5mi transgenic mouse model, in which the majority of CD4⁺ T cells express a diabetogenic TCR derived from the diabetogenic BDC-2.5mi T cell clone [395]. Interestingly, both IV and ID administration of PS-2.5mi-liposomes led to a significant increase in splenocyte numbers as early as 4 days post-treatment, compared to empty PS-liposomes, indicating the expansion of antigen-specific T cells. Indeed, our antigen-specific treatment substantially increased the percentage of **CTLA4⁺ Treg cells** within both the CD4⁺ and CD8⁺ compartments. As observed before, after phagocytosing PS-2.5mi-liposomes, professional APCs in the spleen acquire tolerogenic features [388]. These tolerogenic induction is likely mediated through different mechanisms such as the secretion of anti-inflammatory and pro-resolving molecules that promote Treg cell formation and expansion [357]. The upregulation of CTLA4 expression has been reported to occur on Foxp3⁺ Treg cells when undergoing extensive proliferation rates [470]. CTLA4⁺ Treg cells, recognized as a distinct subset

from conventional FoxP3⁺ Tregs, exhibit enhanced suppressive capacity through mechanisms such as increased IL-10 production, trogocytosis of CD80/CD86 complexes from APCs, and upregulation of PD-L1 on APCs, reinforcing the immunoregulatory loop [35,471–474]. Furthermore, the expression of CTLA4 in Tregs has been shown to be indispensable for immune homeostasis, as it controls the formation of antigen-specific Tfh cells, germinal center, and plasma and memory B cell [475].

Additionally, this experiment confirmed the expansion of FoxP3⁺ antigen-specific **Tr1 cells** after IV administration of PS-2.5mi-liposomes, correlating with other immunotherapies also intravenously administered, and probably likely due to the role of splenic APCs and liver LSECs which produce IL-10 and together with the lack costimulatory molecules, favours Tr1 differentiation [476–478]. On the other hand, tolerogenic DCs are also known to promote **T cell exhaustion**, a process consistent with our observations. We detected an increasing trend in exhausted T cells, characterized by upregulation of immunoregulatory checkpoints such as PD-1 and CTLA4. Furthermore, tolerogenic DCs with the low expression of CD80/CD86 costimulatory markers, along with CTLA4⁺ expression in Treg cells and exhausted T cells, promoted the expansion of the putative non-reversible **anergic T cells**, a phenomenon that became particularly evident at 13 days post-treatment [479–481]. Interestingly, the reversible anergic T cells that persist long-term in the host may have the ability, after restimulation with the antigen, to reverse their phenotype into FoxP3⁺ T cells or Tr1 cell, reinforcing the concept of sustained immune tolerance [482,483]. Regarding effector memory T cells, at 13 days post-administration, we detected an increased percentage of effector memory CD4⁺ T cells. This phenomenon could be beneficial for the establishment of long-term tolerogenic memory, as antigen-experienced CD4⁺ T cells have been reported to maintain plasticity and differentiate into Tr1 cells upon chronic antigen exposure [484–486].

To further expand the complexity to of the tolerance induction mechanisms observed, although not in a proven antigen-specific manner, we detected an increase in certain **Breg subsets**, including the described CD19⁺CD1d⁺LAG3⁺ and the rather unknown CD19⁺FoxP3⁺ subset. Although FoxP3 is not a typical marker for B cells, its expression has been reported in a small subset of Bregs, which are thought to contribute to immune tolerance [487,488]. This process may be synergistically promoted by tolerogenic DCs and various Treg subsets, which preferentially drive Breg differentiation while simultaneously suppressing effector B cell responses. The expansion of these regulatory populations is likely influenced by the balance of co-stimulatory and inhibitory molecules, cytokines, and metabolic enzymes produced

by DCs and Tregs [489]. Additionally, cell-mediated interactions with Treg subsets could play a key role, particularly through CTLA-4, which has been implicated in modulating B cell responses toward a tolerogenic phenotype [490]. Furthermore, these findings raise the question of whether phagocytic B cells, previously identified in other studies, could be directly modulated by PS-liposomes, mimicking apoptotic cell clearance mechanisms [442,491]. Indeed, if PS liposomes could directly interact with B cells and modulate their phenotype towards a tolerogenic phenotype, this would be a major advantage, especially for autoimmune diseases primarily driven by B cells.

The ultimate proof of the antigen-specific immunomodulatory capacity of PS-loaded-liposomes was obtained through *in vivo* studies in animal models of MG and RA, both B cell driven diseases. In either model, therapeutic administration of disease-relevant PS-loaded liposomes significantly ameliorated disease severity [405]. In the case of RA, the antigen-induced arthritis murine model—characterized by synovial inflammation, leukocyte infiltration, pannus formation, and progressive cartilage and bone erosion—was treated intraperitoneally with PS-MCV-liposomes. This, effectively reduced synovitis and local bone erosion compared to control treatment with empty PS-liposomes and notably prevented disease development. Similarly, in the experimental autoimmune MG model, treatment with PS-AChR-liposomes led to a clear reduction in clinical signs severity, accompanied by a significant decrease in serum levels of AChR-specific IgG antibodies when compared to animals treated with either empty liposomes or PS-liposomes loaded with irrelevant peptides. These results shed light on our hypothesis that PS liposomes can directly interact with B cells and modulate them towards a tolerogenic state. However, further research is needed to confirm this hypothesis and to elucidate the potential role of PS liposomes in shaping B cell-mediated tolerance.

Taking together, these results explain the regulatory effect of antigen-specific PS-liposomes induced by tolDCs and highlight the multifaceted and self-reinforcing nature of antigen-specific immune tolerance induced by PS-loaded liposome therapy (**Figure 43**). However, it is essential to better define this phenomenon, especially to be able to detect the desired biomarker for long term tolerance induction, especially as a proof of efficacy in clinical practice. At the present, the determination of whether the immune system has returned to a state of health and the patient has achieved permanent remission can only be made through extended longitudinal observation. In certain cases, patients with RA treated with DMARDs achieve a remission for sustained periods, suggesting that immune homeostasis may be restored at least in the medium term [492]. Nevertheless, definitive biomarkers for immune tolerance

or restored immune homeostasis in patients with immune-mediated inflammatory diseases remain unidentified. While current research primarily focuses on immune system-related biomarkers, expanding this approach to include predictors of disease relapse—such as serological, proteomic, metabolomic, or cellular markers—may enhance clinical success and advance the field.

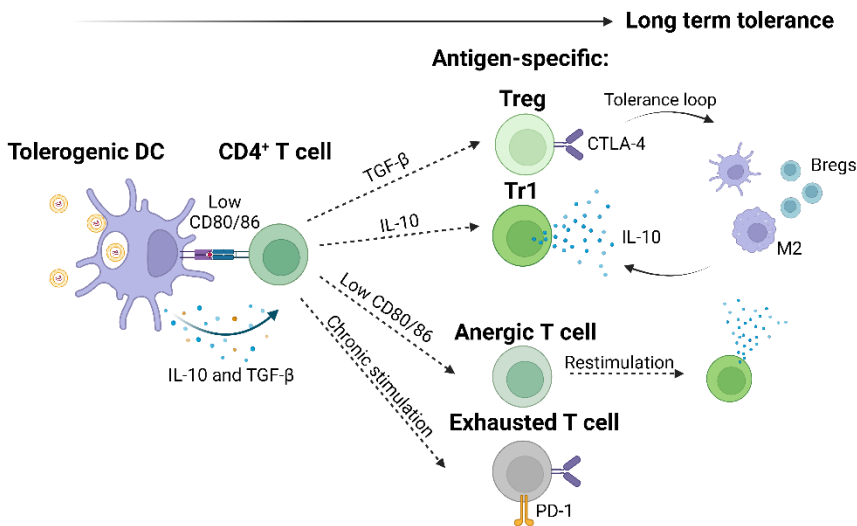


Figure 43. Antigen-specific tolerogenic crosstalk between APCs and T cells following PS-loaded-liposomes treatment. Upon PS-loaded-liposomes efferocytosis by APCs, particularly DCs, these cells acquire a tolerogenic phenotype. The increase of IL-10 and TGF-β anti-inflammatory cytokines, promotes the expansion of antigen-specific regulatory T cell subsets, including FoxP3⁺ Tregs and Tr1 cells, which further sustain immune tolerance through expression of inhibitory molecules like CTLA-4 and secretion of additional anti-inflammatory mediators. These tolerogenic T cells not only suppress effector T cell responses but also contribute to the reprogramming of other APCs, such as Mφ and B cells, toward tolerogenic phenotypes, establishing a positive feedback loop that reinforces immune regulation. Simultaneously, the presence of tolerogenic DCs and the expanding pool of T regulatory cell subsets, fosters the differentiation of Bregs, which suppress proinflammatory B cell responses and contribute to maintaining antigen-specific unresponsiveness. The low expression of CD80/86 co-stimulatory makers on DCs leads to the induction of T cell anergy. Importantly, under antigen re-stimulation, some anergic T cells can transdifferentiate into FoxP3⁺ T cells or Tr1 cell, reinforcing the long-term tolerance. Moreover, repeated antigen exposure under tolerogenic conditions may drive T cell exhaustion, typified by the upregulation of multiple immune checkpoint receptors, thereby further limiting pathogenic effector responses. Figure created using BioRender.

Human model: Immune modulation of phenotypic markers and function on DCs after PS-liposome uptake

After demonstrating that PS-liposomes loaded with β -cell autoantigens and MOG peptides could restore immune tolerance in experimental models of T1D and MS, the potential of this approach as a platform for treating various autoimmune diseases became evident [388,389]. These findings underscore the crucial role of PS-enriched liposomes in driving a tolerogenic response and highlight the efficiency of phagocytic cells in engulfing, processing, and presenting encapsulated peptides in an antigen-specific tolerogenic manner. However, the ultimate goal of this therapy is its translation to human applications. Despite the valuable insights gained from murine models, notable differences exist between the mouse and human immune systems, including variations in APC populations, cytokine signalling pathways, and immune cell distribution [493–495]. Moreover, experimental models are typically established artificially in mice, replicating certain aspects of human autoimmune diseases but lacking their full complexity. Therefore, a critical step in the development of novel immunotherapies is to validate their effects using human immune cells *in vitro*.

Previous studies have already demonstrated the clinical potential of PS-liposomes encapsulating human insulin in T1D patients [390]. In the study, DCs that engulfed these liposomes exhibited a tolerogenic phenotype and antigen-specific functionality, characterized by a reduced capacity to stimulate autologous T cell proliferation. To bridge the positive platform results obtained in mice models and the previous promising results in T1D patients, in **SECTION III**, we sought to assess for the first time the clinical platform potential *in vitro* of PS-loaded liposomes for MG and RA patients by encapsulating AChR and MCV peptides. To facilitate this research, a collaboration was established with Sant Pau and Vall d’Hebron hospitals (Barcelona, Spain) to obtain blood samples from MG and RA patients, respectively. Monocytes were isolated from PBMCs of each patient and successfully differentiated into DCs, achieving optimal yield, purity, and viability. The impact of PS-loaded liposomes on DCs was then evaluated in terms of viability, phagocytic capacity, phenotype, peptide presentation, and their ability to regulate T cell proliferation.

For the first time, we evaluated DC from MG and RA patients to phagocytose their respective PS-loaded-liposomes. Previous results in the context of T1D, showed efficient phagocytosis of PS-Ins-liposomes by human DCs, however, lower levels of capture have been observed in T1D paediatric patients [390,391]. Here we observed comparable phagocytosis rates to those results observed in T1D and healthy control subjects previously, with no substantial differences between MG and RA. As

expected, iDCs, specialized in antigen capture and processing, showed higher phagocytic rates than mDCs due to the loss of endocytic ability during maturation process by the shift of their primary role from antigen capture to antigen presentation [496]. This uptake result correlates with the expression of PS receptors found in immature and mature conditions.

By the **phenotype assessment of DCs**, an immunomodulatory effect of PS-liposomes was validated. First, a positive feedback of PS receptor expression in immature conditions was observed after their uptake. By contrast, PS receptors in mDCs were not upregulated, correlating with the loss of phagocytic ability but probably with an enhanced migratory functionality to focus on their role in T cell modulation. Tolerogenic feature acquisition was evidenced by the downregulation of costimulatory molecules, as CD86 in mDCs, a finding consistent with previous observations in murine DCs and human T1D-derived DCs following incubation with PS-loaded-liposomes [388–390]. However, compared to other tolerised DCs in other studies, no reduction in CD40 expression was observed [497]. This fact aligns with the complexity of defining a uniform tolerogenic DC phenotype, as distinct subsets of tolerogenic DCs may exhibit diverse phenotypic characteristics. Interestingly, a downregulation of the CD54 marker was detected in mDCs that had engulfed PS-liposomes. This reduction in CD54 expression, has been reported in other tolerised DCs, and may contribute to tolerance induction by affecting T cell activation given its fundamental role in immunological synapse formation and stability [498,499]. This decreased expression may lead to less efficient T cell activation or preferentially promote the development of Tregs over effector T cells [500]. Furthermore, the reduction of CD54 and CD86 did not occur when mDCs were cultured with empty PS-liposomes, suggesting a possible effect of the antigen released by the PS-liposomes on this decrease and not of the empty PS-liposome itself. The study of chemokine receptors revealed an enhanced migratory function to lymphoid organs or to the white pulp in the spleen of DCs upon the PS-liposome engulfment [501]. CCR7, the key marker of homeostatic maturation, as observed in other studies with AC, after PS-liposome engulfment upregulated its expression, particularly those DCs treated with NBD-labelled PS-liposomes [502,503]. Similarly, CXCR4 expression was also upregulated, particularly in mDCs. While CXCR4 has not been directly linked to immune tolerance induction, its role in DC migration to lymphoid tissues is well established, as it facilitates trafficking along CXCL12 gradients, which are abundantly present in lymphoid tissues and T cell zones [504]. Moreover, CXCR4 has been extensively studied in the context of solid tumours and hematologic malignancies, where its overexpression has been linked to immune suppression mechanisms

exploited by cancer cells [505]. Recent studies have also demonstrated that targeting a minor allosteric pocket of CXCR4 induces an immunomodulatory effect, influencing TNF-I levels through interactions with the TLR pathway [506,507]. Additionally, supporting the tolerogenic potential of PS-liposome-treated DCs, the upregulation of immunoregulatory markers, such as DC-SIGN and PD-L1, was prompted [508,509]. It is worth noting that the increase of PD-L1 was not shown when mDCs were cultured with PS-empty-liposomes, suggesting a possible effect of the encapsulated antigen in this decrease rather than the PS-liposome alone.

As part of our investigation into the mechanism of action of our therapy, we aimed to check the specific **antigenic presentation** of the encapsulated peptide in PS-loaded-liposomes, to ensure the antigen-specific immunomodulatory effect of the DCs engulfing them. To achieve this, we conducted a proteomic analysis using moDCs derived from healthy donors recruited from the Sanquin Blood Supply Centre (Amsterdam, The Netherlands). These DCs were first treated with PS-AChR-liposomes and PS-SPACER-liposomes, designed to target MG and iTPP, respectively, and then matured. In parallel, DCs were also incubated with the corresponding free peptide, given the established ability of mDCs to uptake free peptides from the extracellular environment and present them via HLA class II molecules [510]. Since MG has been associated with different HLA alleles depending on ethnic background, whereas iTPP is strongly linked to the HLA-DRB1*11 allele, a predictive HLA presentation analysis was performed for both peptides [101–104,189]. The differentiation of monocytes into DCs was efficient, and as anticipated, PS receptor upregulation, was observed in DCs exposed to PS-loaded-liposomes, confirming their successful uptake. However, due to the limited number of control donors and their intrinsic heterogeneity, no significant differences were detected in the expression of other surface markers. Notably, the upregulation of CCR7 and, in particular, CXCR4 after PS-liposome uptake was consistently observed across all control donors.

The proteomic data generated from all controls and conditions used shed extensive insights. As previously observed by other authors, total peptide count was always higher in HLA-DR than HLA-DQ [511]. In addition, the analysis of the **HLA-DR immunopeptidome** in control conditions revealed substantial heterogeneity in peptide presentation among donors, potentially influenced by individual genetic variability, molecular changes during differentiation, or variable responses to maturation stimuli [512]. Peptides that predominantly originate from endogenous proteins rather than exogenous sources [513,514]. In line with this, our data consistently showed the presentation of self-derived peptides, including albumin, the most abundant serum protein, heat shock cognate protein, probably as a result of

LPS induced stress during maturation, apolipoprotein A-I, histone H4, and pro-low-density lipoprotein receptor-related protein 1, across all conditions. These findings highlight the homeostatic nature of the MHC class II immunopeptidome, supporting the concept that the baseline presentation of self-antigens play a role in tolerance induction, a mechanism that has been proposed as a therapeutic strategy for different autoimmune settings [515–518]. The extensive dataset obtained from this study offers valuable opportunities to further investigate MHC-peptide interactions, assess potential HLA type or haplotype preferences, and explore the cleavage specificity of proteases involved in HLA-DR and HLA-DQ ligand processing. Additionally, characterizing the tolerogenic presentation of naturally processed peptides from other self-proteins could provide further insights into immune tolerance mechanisms.

While some studies propose that efferocytosis of ACs leads to the suppression of MHC class II-mediated antigen presentation [519,520], others support the concept of homeostatic presentation of self-antigens derived from ACs as a mechanism for inducing immune tolerance [521–523]. Although in the present study we did not use AC directly, our findings provide new insight into the underlying mechanisms governing antigen processing and presentation in the context of efferocytosis-like processes. In the conditions where **PS-loaded-liposomes** were used, we successfully detected SPACER peptides presented by both HLA-DR and HLA-DQ molecules. When comparing these findings to the condition with free ADAMTS13 SPACER peptide, we observed a higher number of SPACER peptides in the free peptide condition than in the PS-SPACER-liposome condition. This discrepancy is likely attributable to differences in the final peptide concentration, as when administered directly to the culture medium the peptide had a final concentration of 20µg/ml, however, administering the PS-SPACER-liposomes the peptide was diluted to a final concentration of 0.013µg/ml. Regarding the AChR peptide, although in some conditions was detectable, most of the conditions, either with free peptide or PS-AChR-liposomes showed no or low counts of the peptide of interest. Given the successful detection of SPACER peptide and the absence of AChR peptide even in free peptide conditions, we hypothesize that the AChR peptide may be lost at some stage during the mass spectrometry workflow. This loss is likely due to its physicochemical properties. Specifically, the SPACER peptide exhibits a basic nature, allowing it to remain soluble in acidic media, whereas the AChR peptide is acidic and tends to precipitate under these conditions. Additionally, the net charge of SPACER peptide is positive at pH 7, whereas AChR peptide is negatively charged and has been shown to degrade at high temperatures. These differences in charge, solubility, and stability

may significantly influence their behaviours under identical experimental conditions. To confirm this hypothesis, adjustments to the experimental protocol would be necessary. Potential modifications including optimizing buffer compositions, modifying temperature conditions, or employing alternative mass spectrometry strategies to enhance peptide recovery and detection [514].

Then, having checked that DCs are able to present the encapsulated peptide, we assessed the functionality of tolerogenic DCs after synapsing with autologous T cells. Previous results with T1D patients showed promising tolerogenic effect of those DCs treated with PS-Ins-liposomes since could impair the proliferation of CD8⁺ T cell subset, pointing out the reduction of T cell cytotoxicity [390]. Here, to assess this tolerogenic effect of DCs after PS-loaded-liposomes engulfment, we performed again **autologous T cell proliferation assay**, this time with cells from MG and RA patients. It is noteworthy to mention that two patients of MG were excluded from this study due to exacerbated proliferative ratios, likely due to the presence of the combination of the HLA alleles DQA1*03 and DQB1*03 forming the HLA-DQ8 heterodimer. This HLA-DQ8 is associated with strong autoimmune reactions and MG disease in different geographical ethnicities [101,524,525]. DQ8 may stem from structural and functional properties that enhance autoantigen presentation and T cell activation, indeed, if our AChR encapsulated peptide strongly binds to DQ8, this could override the tolerogenic effect of PS-liposomes in this assay by sustaining T cell activation. However, this does not determine the non-successful fate of patients carrying HLA-DQ8 haplotype since the tolerogenic loop generated in the spleen and the liver could reverse this strong binding and activation. Nevertheless, more studies should be carried out to better understand these phenomena, in addition, to ensure security in future clinical trials, patients carrying HLA-DQ8 haplotype should be excluded.

Regarding the responder's patients, ultimately, we assessed that those mature DCs pulsed with the free peptide and treated afterwards with PS-loaded-liposomes, were able to successfully impair T cell autologous proliferation in both, CD4⁺ and CD8⁺ compartments. This suggests that even after a maturation stimulus those tolerogenic DCs induced by PS-liposomes can maintain their tolerance induction. The inability of empty PS-liposomes treated mDCs to impair CD4⁺ and CD8⁺ T cell autologous proliferation supports the tolerogenic antigen-specific effect through the encapsulated peptide presentation. Furthermore, looking at the antigen-specific response in MG samples addresses the question of whether DCs can present the encapsulated AChR peptide. However, we are aware of the complexity of the mechanism governing the induction of T cell proliferation, and more studies should be performed to corroborate the antigen-specific tolerance induction. Adding

markers to the cytometry panel like CD25, FoxP3, CD45RO/RA, and intracellular cytokines would help to discern the mechanism of why tolerogenic DCs can impair this proliferation. Cytokines produced during the autologous proliferation studies also would shed light on Th profiles induced, helping to better understand how DCs modulate this phenomenon.

When available, synovial fluid from RA patients was collected, and **SFMCs** were isolated. From these, CD14⁺ cells were purified, which likely comprised primarily by proinflammatory monocyte subsets, moMφ and in a less percentage, tissue-resident Mφ [526–528]. Little is known about this poorly studied fraction of the synovial fluid, which makes this part of the study very relevant to the effect of PS liposomes in an inflammatory region. The high expression of CD11c on these cells after 6 days of modulation with GM-CSF and IL-4, suggest that most of the initial monocytes transitioned to DCs, however, probably some of the Mφ persisted undifferentiated, although in low percentages. The synovial fluid derived DCs were subsequently incubated with PS-MCV-liposomes under the same conditions as blood-derived monocytes, with the objective of evaluating their response to PS-liposome-mediated tolerogenic modulation, despite originating from an hyperinflammatory environment [526,529]. Remarkably, exposure to PS-MCV-liposomes had a similar effect to those observed in moDCs derived from blood, particularly through the upregulation of PS receptors. Conversely, in terms of costimulatory molecules and HLA-DR expression, an increasing trend was observed in both immature and mature conditions following PS-MCV-liposome exposure, possibly reflecting the prior inflammatory state of these cells [527,528,530]. Similarly, the expression of CCR7, CXCR4, DC-SIGN and PD-L1 followed the same trend as monocyte derived DCs from PBMCs, despite the initial proinflammatory. These findings suggest that, even in a highly inflammatory state, PS-MCV-liposomes can modulate certain aspects of DC phenotype in a tolerogenic direction. Due to the small sample size, more studies are required to validate these preliminary findings and to provide a more comprehensive understanding of their therapeutic potential in the immune cell subsets present in synovial fluid.

Following the phenotypic characterisation, putative DCs isolated from synovial fluid were functionally assessed by coculture with the CD14⁺ cell fraction of SFMCs, which is naturally enriched in autoreactive T cells [531]. The objective was to evaluate the capacity of these DCs to modulate T cell proliferation, as previously done with DCs and T cells from blood. Notably, synovial-derived DCs—whether maintained under immature conditions or subjected to maturation—demonstrated a capacity to suppress both CD4⁺ and CD8⁺ T cell proliferation upon engulfment of PS-MCV-

liposomes. A reduction in CD8⁺ T cell proliferation was observed in the condition with matured DCs, however, in iDCs induced comparatively higher T cell proliferation. While this may appear contradictory, several hypotheses may explain this observation, including the expansion of Tregs, the lack of inhibitory checkpoints in comparison to matured DCs or the synovial T cell pre-activation that produce exhaustion or anergy matured DCs but not in immature. Further experimental approaches are needed to better understand the DCs-T cell crosstalk in the synovial fluid, such as cytokine profiling of the coculture supernatants, functional blockade of immune checkpoints to assess the recovery of T cell responses, and antigen-specific tracking using peptide-MHC tetramers to distinguish between specific and bystander T cell activation.

PS-liposome interaction with human B lymphocytes

Additionally, to further investigate the interactions between PS-liposomes and other immune cell subsets within PBMCs, we conducted phagocytosis assays and analysed cytokine production, providing further insights into the broad tolerogenic mechanisms of PS-liposomes. The clearance of apoptotic cells is a fundamental physiological mechanism for maintaining immune tolerance, traditionally attributed to professional phagocytes such as M ϕ and DCs [532]. However, our findings suggest that PS-liposomes not only interact with professional phagocytes but also **engage B cells *in vivo* (SECTION I)**. To our knowledge, there is no NP that can directly interact with B cells. Therefore, in this doctoral thesis for the first time the interaction of PS-liposomes with B cells has been reported [405]. It is worth noting that despite showing lower interaction ratios than classical phagocytic cells, since they are found in such abundance in spleen and liver, the total number of B cells interacting with PS-liposomes equals the number of interacting phagocytes, and should therefore not be underestimated. Aiming to investigate this phenomenon in humans, PBMCs from healthy donors and MG and RA patients were incubated with fluorescently labelled NBD-PS-liposomes, revealing a small but consistent percentage of circulating B cells capable of interacting with PS-liposomes. This 4% of interacting cells, as literature indicates, probably are circulating marginal zone B cells or B1 cells, subsets with the proven ability of interact with apoptotic cells and which represent the 1 to 9% of the total circulating B cells in humans [533–535]. It should be noted that these B cells, positive for the NBD fluorescence of PS-liposomes, exhibited an enhanced tolerogenic phenotype, as evidenced by the upregulation of IL-10 and TGF- β . This supports the hypothesis that PS-liposomes influence B cell-mediated immune modulation, a phenomenon previously described in AC and B cell interactions [376,533]. Given the literature describing phagocytic B cells, we sought to further

characterize the fate of PS-liposomes upon interaction with B cells. By successfully labelling PS-loaded-liposomes with the pH-sensitive dye pHrodo, we confirmed that PS-liposome internalization was not a mere surface interaction but rather a phagocytic event. The acidification of PS-liposomes within B cells, indicative of lysosomal localization, confirms that PS-liposomes are internalized and processed within the lysosomal compartment, where pH ranges between 4.5 and 5 [536].

At this point, we explored the nature of the B cell and PS-liposome interaction, particularly because primary B cells were traditionally thought to lack phagocytic capacity. While much of the literature supports the notion that B cell interaction with apoptotic material is mediated via BCR or TLR9 signalling [376,533], recent studies suggest that B cells in other species are capable of engulfing large particles, hinting at the presence of specific receptors [537–539]. However, this function remains poorly characterized in humans and mice, warranting further investigation. Our flow cytometric analysis of PS receptor expression in human peripheral blood B cells revealed for the very first time detectable levels of Axl, integrin $\alpha\beta v5$ and TIM-1, with expression levels comparable to those observed in monocytes. These findings suggest a potential role for these receptors in PS recognition and liposome internalization by B cells. Axl and integrin $\alpha\beta v5$ are key PS-recognizing receptors typically associated with the engulfment of apoptotic cells by professional phagocytes. Although evidence for their expression in primary human B cells is limited, Axl has been shown to be upregulated in certain B cell malignancies, such as chronic lymphocytic leukaemia [540]. Of particular interest was the expression of TIM-1 on B cells. TIM-1 expression has been strongly correlated with Breg functionality, as TIM-1⁺ Bregs show to exhibit an 8- to 20-fold enrichment for IL-10 production across multiple B cell subsets, including transitional, marginal zone, follicular, and CD1d^{hi}CD5⁺ B cells [541,542]. Moreover, circulating TIM-1⁺ human B cells have been identified and TIM-1 has recently been proposed as an immune checkpoint for improving anti-tumoral therapies [543,544]. Separately, other studies advocate a TIM-1-independent contact between B cells and ACs, resulting in the secretion of natural self-reactive antibodies and IL-10 [376,378].

When PBMCs were cultured with PS-AChR-liposomes, Axl surface expression was downregulated in both monocytes and B cells. This could be explained by PS-liposome opsonization via GAS6 and subsequent Axl binding and internalization—a mechanism well-described in professional phagocytes and consistent with the efferocytosis-like uptake observed in monocytes. Notably, when we compared between those NBD positive or negative subsets, in both cell subsets, those that had internalized PS-liposomes expressed higher levels of PS receptors, suggesting that

receptor engagement facilitates uptake. However, despite a similar increase in overall PS receptor expression in interacting B cells, Axl expression was not downregulated as observed in monocytes. This differential behaviour hints at a distinct internalization mechanism in B cells—potentially independent of classical efferocytosis pathways. It is conceivable that PS recognition in B cells involves unique or alternative signalling cascades, possibly influenced by TIM-1-mediated processes.

Importantly, TIM-1 ligation is known to promote IL-10 secretion in Bregs [541]. The hypothesis that TIM-1⁺ B cells can interact with and likely internalize PS-liposomes suggests a previously undescribed mechanism by which TIM-1⁺ B cells contribute to AC clearance and immune tolerance induction. Supporting this notion, TIM-1-deficient mice exhibit a deficiency in IL-10 production by Bregs and fail to produce IL-10 following apoptotic cell uptake. Furthermore, treatment with AC ameliorates EAE in wild-type mice but not in TIM-1-deficient hosts, reinforcing the hypothesis that PS-liposomes may induce a similar immunomodulatory response [545,546]. Following this line of results, unpublished data from Ahead Therapeutics have shown how *in vivo* B cell blockade with anti-CD20 inhibits the effect of PS liposomes in murine models, demonstrating the important role of B cells in achieving tolerance. Despite these promising findings, further research is required to fully characterize the nature of PS-liposome internalization by B cells, determine their antigen-presenting capacity, and study their impact on T cell responses to clarify their role in adaptive immune regulation upon PS-liposome interaction.

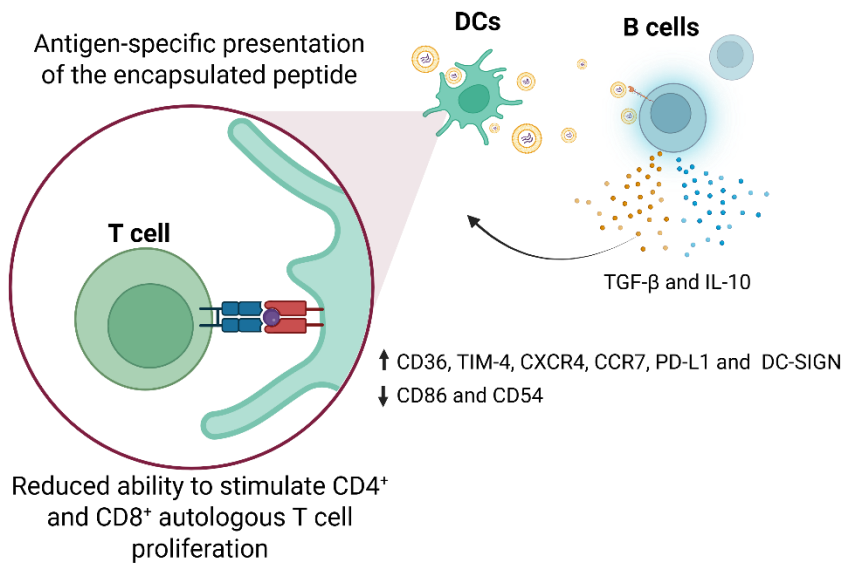


Figure 44. Effect of PS-loaded liposomes on human DCs phenotype and function, and on B cell cytokine production. Upon engulfment of PS-loaded liposomes, DCs exhibit a tolerogenic phenotypic shift characterized by the upregulation of phosphatidylserine receptors CD36 and TIM-4, chemokine receptors CXCR4 and CCR7, and tolerogenic markers PD-L1 and DC-SIGN, and by a downregulation of the co-stimulatory molecules CD86 and CD54. Simultaneously, DCs are capable of processing the encapsulated peptide and presenting it via MHC class II molecules, thereby modulating the antigen-specific recognition of autoreactive T cells. This results in a reduced ability of DCs to stimulate autologous CD4⁺ and CD8⁺ T cell proliferation. In parallel, B cells that internalize PS-liposomes increase their production of the anti-inflammatory cytokines IL-10 and TGF- β , contributing to the establishment of an immunoregulatory loop that reinforces the tolerogenic crosstalk among DCs, T cells, and B cells. Figure created using BioRender.

Regulatory safety of PS-AChR-liposomes

The transition from preclinical research to clinical trials represents a critical step in the development of novel immunotherapies, requiring rigorous regulatory assessment to ensure safety, efficacy, and quality. Both the European Medicines Agency (EMA) and the FDA establish stringent guidelines that govern the preclinical evaluation of advanced therapeutic products, including nanomedicine-based interventions such as our PS-loaded-liposomes. These regulatory frameworks need comprehensive *in vitro* and *in vivo* studies to characterize the mechanism of action, pharmacokinetics, biodistribution, immunogenicity, and potential toxicity, ensuring a well-defined risk-benefit profile before first-in-human administration. In **SECTION IV**, we focused on some of the preclinical experiments to demonstrate the safety,

tolerability and proven efficacy of our **PS-AChR-liposomes**, the leading asset of Ahead Therapeutics.

Ensuring the safety and biocompatibility of PS-liposomes is crucial for their clinical translation. Previous studies of our group have already demonstrated that PS-liposomes administered IP at high doses for 14 consecutive days in pre-diabetic NOD mice were well-tolerated, with no signs of systemic toxicity, as confirmed by biochemical blood analysis [547]. Furthermore, IV administration in BDC2.5mi NOD mice showed that PS-2.5mi-liposomes were also well-tolerated over time, suggesting an overall favourable safety profile. However, further preclinical and clinical studies are required to confirm their long-term safety in humans. Since blood is the first tissue in contact with NPs following IV injection, a key concern in nanomedicine development is their potential interaction with erythrocytes, which could lead to oxidative stress, haemolysis, morphological alterations, membrane deformability impairment, or haemoglobin structural changes [548,549]. Some studies suggest that phosphatidylserine induces blood coagulation, jeopardizing the IV administration of PS-liposomes [550]. To this end, a **hemocompatibility study** was performed using blood from healthy controls that was incubated for different times, ranging from 5 to 120 minutes, at different concentrations of PS-AChR-liposomes. The morphological profile of each donor was evaluated with the use of the acoustic focusing technology on the cytometer, described as a novel methodology to evaluate the quality of erythrocytes [551]. This method enables to detect possible structural changes in erythrocytes such as size or shape variations like echinocytes (spiky cells) or stomatocytes (cup-shaped cells) features indicative of oxidative stress, membrane damage, or cytoskeletal disruption [552]. Importantly, the morphological profile of erythrocytes remained unchanged across all conditions, suggesting no significant alterations in RBC deformability or viability. The preservation of normal RBC structure ensures optimal blood viscosity and microcirculatory perfusion, which is critical for *in vivo* applications of PS-liposomes. Another essential factor in blood compatibility is the interaction between nanoparticles and plasma proteins, which can trigger coagulation pathways by inducing plasma protein adsorption, coagulation factor activation, platelet adhesion, or aggregation. However, total erythrocyte singlet counts did not indicate any cell aggregation after long periods of direct exposure to PS-AChR-liposomes, regardless of concentration. The absence of clot formation suggests that PS-liposomes do not induce thrombogenic effects, a crucial safety criterion for IV administration. Furthermore, given that haemoglobin is essential for oxygen transport, any oxidative modifications in its structure could compromise oxygen delivery, potentially leading to fatigue, organ dysfunction, or anaemia [553].

Using light scattering analysis at 405 nm, haemoglobin oxidation was monitored and found to remain stable across all tested conditions, further supporting the hemocompatibility of PS-AChR-liposomes. Since nanoparticle-induced haemolysis is influenced by factors such as surface charge, particle size, and concentration, the osmotic fragility of RBCs was also evaluated under different PS-AChR-liposome exposure conditions [554,555]. No increased erythrocyte susceptibility to haemolysis was observed, reinforcing the safety of PS-liposomes when in direct contact with human blood.

On the other hand, **cytokine release syndrome (CRS)** is a life-threatening systemic inflammatory response that has been reported following intravenous administration of various biological therapies, including CAR-T cell therapies, monoclonal antibodies, and other immunomodulatory agents [556,557]. CRS can also arise as part of a dysregulated immune response to infections, such as in severe COVID-19 cases [558]. Clinically, CRS is characterized by high fever, hypotension, hypoxia, capillary leakage, coagulopathy, respiratory distress, and severe organ dysfunction, which typically manifest during or immediately after drug administration [559]. Although the precise cellular mechanisms of CRS can vary depending on the triggering agent, its pathogenesis is primarily driven by excessive cytokine release following the activation and cytotoxic activity of monocytes, M ϕ , and various lymphocyte populations [560]. In order to improve CRS risk prediction and support first-in-human clinical trials, *in vitro* cytokine release assays have been widely adopted, providing insights into potential proinflammatory effects before clinical administration [561,562]. Based on the mechanism of action of PS-AChR-liposomes, we sought to evaluate their ability to induce CRS-like cytokine responses using a whole blood coculture model from healthy donors. For a positive control, we selected LPS, a well-established potent inducer of cytokine release, since other commonly used positive controls, such as TGN 1412 (anti-CD28 superagonist) and Campath (anti-CD52 antibody), were not accessible for our study [563–565]. CRS models consistently highlight IL-6 as a central cytokine in its pathophysiology, with other key inflammatory mediators, including TNF- α , IL-1, IL-2, and IFN- γ , contributing to tissue damage and systemic inflammation [566–568]. Notably, none of these cytokines, nor any other proinflammatory mediators analysed, were induced following PS-AChR-liposome exposure in any tested condition. Additionally, GM-CSF, a cytokine implicated in monocyte-dependent CRS responses, remained unchanged, further supporting the absence of an inflammatory activation signature upon PS-liposome exposure [569]. Similarly, chemokines such as CCL3 and CCL4, which have been associated with CRS onset, did not show any alterations in serum samples

exposed to PS-AChR-liposomes [570,571]. While liposome-based therapies have previously been evaluated in clinical trials without evidence of CRS induction, regulatory agencies generally require science-based cytokine release risk assessments as part of nonclinical safety evaluations for novel IV administered NP formulations. Since PS-AChR liposomes did not show proinflammatory cytokine release in our study, these preclinical safety data help guide first-dose selection and dose-escalation strategies for the future clinical trial. However, as with all *in vitro* cytokine release assays, extrapolations to *in vivo* human responses should be made with caution.

Taken together, our results provide strong evidence that PS-AChR-liposomes are safe for IV administration, showing excellent hemocompatibility, with no adverse effects on erythrocyte morphology, aggregation, haemoglobin oxidation, or haemolysis, and critically, no CRS induction. These findings further support their potential for clinical translation, reinforcing their favourable safety profile as an antigen-specific therapy for autoimmune diseases.

A **potency assay** is a quantitative analytical test designed to measure quantitatively the biological activity of a product, ensuring it is manufactured with consistent efficacy and functional integrity. According to the EMA, a potency assay should provide an accurate, reliable and consistent demonstration of the biological activity of the active ingredient, and its design must be scientifically justified, reflecting the mechanism of action of the therapeutic [572]. Similarly, the FDA defines potency as the specific ability or capacity of a product to achieve its intended biological effect, highlighting the need for potency assays to be validated and integrated into the overall quality control strategy for biologics and advanced therapies [573]. Potency assays play a critical role in both preclinical and clinical development, serving as a quality control measure to ensure that each manufactured batch of a therapeutic product maintains its expected biological activity. In preclinical studies, these assays help to characterize the product, support dose selection, and establish proof-of-concept before advancing to clinical evaluation. In the context of clinical trials and eventual commercialization, a well-validated potency assay becomes an essential regulatory requirement, as it ensures batch-to-batch consistency, safeguards product efficacy, and provides a basis for lot release specifications [574]. However, determining the biological activity of immunotherapies can be challenging, as these products often rely on complex immune mechanisms that are poorly or incompletely understood. Biological assays based on cell culture models are commonly used for potency testing, as they enable the measurement of biochemical or physiological responses at the cellular level.

A comprehensive understanding of the cellular biology underlying these mechanisms is essential for defining relevant biological effects. In the context of PS-loaded-liposomes, initial efforts to assess their phenotypic effects on DCs, unexpectedly revealed a precise dose-response relationship in the expression of the TOLX surface marker, particularly in matured DCs derived from MG and RA patients.

The observed dose-dependent TOLX upregulation in mDCs prompted us to explore its potential application in the development of a potency assay. However, due to the complexity and time-consuming nature of monocyte-derived DC protocols, alternative cell models were investigated to establish a more robust, reproducible, and time-efficient potency assay. Given that B cells are capable of internalizing PS-AChR-liposomes and responding in a tolerogenic manner, an immortalized B-cell line was initially tested for its suitability in potency testing [408]. After a screening, we observed that TANOUE cell line demonstrated significant phagocytic activity and a dose-dependent upregulation of TOLX. However, the loss of response as the cell culture aged represented a challenge, and the need to search for a more stable and reliable cellular models.

PBMCs were ultimately selected as a viable alternative due to their ease of collection and the availability of commercial PBMC sources from healthy donors, ensuring a consistent and reproducible cell supply. By systematically optimizing key parameters, including cell concentration, density, incubation time, and dilution range, a robust potency assay was established using B cells from PBMCs. The final assay design, based on cell viability, CD19 expression, and TOLX surface marker analysis, allowed for the generation of a dose-response curve by applying a four-parameter logistic (4-PL) regression model, enabling the calculation of the IC₅₀ for each batch of PS-AChR-liposomes. However, despite these advancements, further development, optimization, and ultimate validation of the assay remain necessary. Factors such as evaporation control, pipetting techniques, plate design, number of replicates, and dilution preparation must be refined to ensure maximum reproducibility and accuracy. Additionally, data analysis methods require optimization, including curve fitting using alternative models such as five-parameter logistic (5-PL) regression, outlier removal, establishment of sample acceptance criteria, and continuous monitoring of relative potency across different batches.

In conclusion, a robust and reproducible potency assay has been successfully developed, providing a valuable tool for assessing the biological activity of PS-AChR-liposomes. Once fully validated, this assay will be instrumental in ensuring batch-to-

batch consistency, supporting regulatory approval, and facilitating the clinical translation of PS-loaded liposomes for therapeutic use.

Future steps for clinical translation of PS-autoantigen-loaded-liposomes

In this study, we established that autoantigen-loaded PS-liposomes hold strong potential for restoring immune tolerance in an antigen-specific fashion of various autoimmune diseases, including T1D, MG or RA. By emulating the natural exposure of PS on the surface of apoptotic cells, these liposomes function as biomimetic nanocarriers that engage physiological mechanisms associated with efferocytosis and tolerance induction. While numerous antigen-specific immunotherapies are currently under preclinical development, aimed at re-establishing tolerance to disease-relevant autoantigens, several factors remain critical for success which should be always carefully considered. These include the compatibility of the delivery system, the method of antigen display, the design, the scalability of the production method and, perhaps most importantly, the choice of antigen. Our preclinical safety assessments support the suitability of this platform for IV administration, with consistent findings indicating biocompatibility and absence of toxicity. Other ASIT NPs therapies under development use this delivery system as a mechanism of action, since targeting spleen and liver phagocytes successfully contributes to tolerance induction [284,288,291,299]. Although some strategies directly target specific subsets of phagocytes and rely more on the reprogramming process through the Treg modulation by FoxP3⁺ Tregs or Tr1 cells [284]. Our therapy not only induces the expansion of these two subsets of Tregs that also permits the interaction of PS-liposomes with other relevant immune cell subsets, such as B cells, key contributor in tolerance establishment. Our approach differs from every other ASIT technology by leaning on the induction of the switch of the B cells interacting with our liposomes to a regulatory phenotype. That is, we potentially reduce the activation of the B cell compartment and use their APC activity to boost the tolerance signal. However, the specific role of B cells in this tolerogenic loop should be further studied. Regarding the design and the antigen display, all materials used in our formulation are naturally biodegradable in the human body, and data from biodistribution studies, *in vivo* capture, long-term administration in murine models, and *in vitro* assays using human cells corroborate the non-toxic, hemocompatible nature of the formulation, with no evidence of CRS or adverse immunological activation. Unlike more complex approaches that rely on multicomponent carriers, protein conjugation, bacterial plasmid vectors or *ex vivo* manipulation of patient cells, our PS-liposome formulation offers a simple and scalable manufacturing process without bioaccumulation, toxicities, immunogenic potential or *ex vivo* complex processes. Comprising only

three lipid components and encapsulating selected antigens, these liposomes are cost-effective and easily modifiable for different autoimmune settings. In comparison to other ASIT therapies, our PS-liposomes lack PGLA or PLG molecules, although being typically considered safe, some studies point out that the inadequate replications of PLGA may result in common mechanisms of toxicity seen with nanoparticles, including inflammation and oxidative stress [575]. Importantly, the absence of PEGylation in our system is also advantageous in light of emerging concerns about pre-existing anti-PEG antibodies, particularly those induced by COVID-19 mRNA vaccines, which could otherwise compromise the efficacy of PEG-containing therapies [576,577]. Despite the advantages of antigen encapsulation in shielding cargo from enzymatic degradation, antigen selection remains a critical consideration since not all strategies can guarantee a broad response by all patient's TCRs. Although the PS-liposome platform is HLA-independent, the decision between using short peptides or full-length proteins is not trivial. While strategies using single peptides require adjustments to the patient's HLA haplotype, the use of full protein antigens is full proteins are more universally applicable but pose challenges in terms of encapsulation efficiency, forcing a trade-off between encapsulation efficiency and the peptide of choice. Hence, until personalised medicine becomes the order of the day, careful peptide choice remains essential to maximize HLA coverage across patient populations. Alternatively, patient stratification based on HLA typing could serve as halfway strategy to tailor treatment more effectively. Finally, it is noteworthy to mention that PS-liposomes do not act as broad immunosuppressants but rather reprogram the immune system while preserving its overall functionality, enabling the induction of durable immunological responses. A central challenge that remains for clinical translation is determining whether this reprogramming leads to a transient or sustained state of tolerance, potentially necessitating intermittent, long-term administration. Although other ASITs have shown effects lasting several months to a year in clinical trials, interpretation of these outcomes must consider the fluctuating nature of autoimmune diseases, and the limitations associated with short-term follow-up periods [283,578].

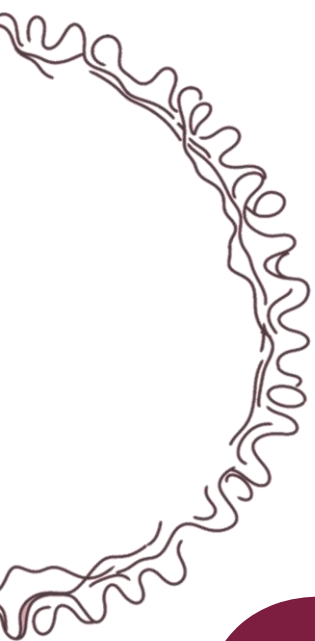
It is important to recognize that in some autoimmune conditions, the mere re-establishment of tolerance may be insufficient for therapeutic efficacy. For instance, in T1D, once the clinical onset has occurred, residual β -cell function is often permanently impaired. In such cases, identifying **immunointervention optimal checkpoints** such as the "honeymoon" phase —a transient period following diagnosis during which some endogenous insulin production is still present—, is crucial to maximize the impact of tolerance-inducing interventions [579]. If

administered beyond this optimal window, combining PS-liposomes with regenerative therapies could represent a viable strategy to both restore and sustain endogenous insulin production. A similar concept applies to RA, where a defined "window of opportunity" exists early in the disease course, often within the first few months of symptom onset [580,581]. Although there is currently no universally accepted consensus among clinicians regarding the categorization of individuals at risk, the term pre-RA is commonly used to describe patients who exhibit early signs of immune dysregulation without fulfilling the full. These individuals are often characterized by the presence of ACPAs as well as evidence of subclinical synovitis even in the absence of clinical joint swelling or pain. Targeting pre-RA patients with immunomodulatory or disease-modifying therapies during this stage may help to modulate the aberrant immune response, reduce systemic inflammation, and ultimately delay or even prevent the progression to overt RA. Based on this strategy, current clinical trials are increasingly focused on defining the risk factors, biomarkers, and optimal therapeutic strategies for this at-risk population [582–584].

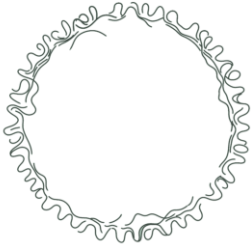
Despite the broad therapeutic potential of PS-liposomes as a platform, the development of any such technology must begin by targeting a specific disease. In this context, MG was selected as the **lead indication** within the development pipeline of Ahead Therapeutics. MG is a prototypical antibody-mediated autoimmune disease, predominantly driven by autoantibodies targeting well-characterized antigens at the NMJ. In approximately 85% of MG cases, the autoantigen is the AChR, enabling precise antigen selection for encapsulation within the PS-liposome formulation [112]. One of the key advantages of MG as a target for ASIT is the availability of reliable biomarkers, such as serum autoantibody titers and quantifiable clinical symptoms including fluctuating muscle weakness and fatigability [585]. These features facilitate the longitudinal monitoring of therapeutic efficacy and immune modulation. Furthermore, MG does not typically cause irreversible neuromuscular damage in its early stages, and symptomatic recovery is often achievable upon effective immunomodulation. This biological reversibility enhances the potential for therapeutic benefit from tolerance-inducing interventions. Importantly, MG is also thought to possess a window of opportunity, particularly during the initial phase following diagnosis or during seroconversion in individuals at risk. During this phase, autoreactive immune responses are not yet fully established, increasing the likelihood of successfully restoring immune tolerance [586]. For these reasons, MG has emerged as one of the most actively pursued autoimmune diseases for the development of next-generation immunotherapies. Over the last seven years, a total of five novel immunomodulatory agents have been approved in the United

States for the treatment of MG, including complement component C5 inhibitors and Fc gamma receptor (FcγR) or neonatal Fc receptor (FcRn) antagonists [143–145,587,588]. In parallel, several investigational therapies are under development, such as CAR-T cell therapies targeting TNFRSF17, inhibitors of the TNFSF13 cytokine family, and CLCN1 chloride channel modulators [589–591]. These therapeutic strategies aim to reduce B cell maturation and survival, diminish pathogenic antibody production, or enhance neuromuscular function. While these innovations have significantly improved symptom management and quality of life for many patients, they do not fundamentally correct the underlying immune dysregulation. Consequently, the disease remains chronic and incurable. This therapeutic gap underscores the urgent need for approaches capable of restoring long-term immune tolerance [592]. In this context, we should remember that ASIT strategies—such as those employing PS-liposomes—represent a promising frontier in the quest to achieve durable remission or even a cure for MG.

To further advance the translational potential of PS-liposomes, **upcoming research** efforts will aim to underscore the immunomodulatory effects of PS-liposome on other immune cells beyond DCs. For instance, bulk and single-cell RNA sequencing (scRNA-seq) of sorted immune cell subsets can reveal gene expression profiles and cellular heterogeneity associated with tolerogenic reprogramming. Complementary epigenetic assays such as ChIP-seq or CUT&RUN targeting key transcription factors involved in immune tolerance—such as PPARγ, AhR, FoxP3, Blimp-1, and IRF4—could uncover regulatory networks driving this phenotype. In parallel, liquid chromatography and mass spectrometry (LC-MS/MS) or nuclear magnetic resonance (NMR)-based metabolomic profiling of treated cells or culture supernatants may help identify metabolic signatures associated with regulatory or anti-inflammatory function. Ultimately, integrative multiomic analyses that combine transcriptomic, epigenetic, proteomic, and metabolomic datasets will be essential to construct predictive models of immune tolerance and uncover master regulatory pathways. These insights could as well inform future clinical development and optimization of the PS-liposome platform.



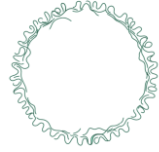
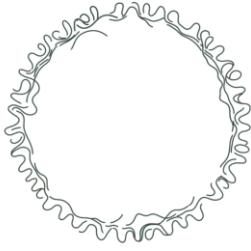
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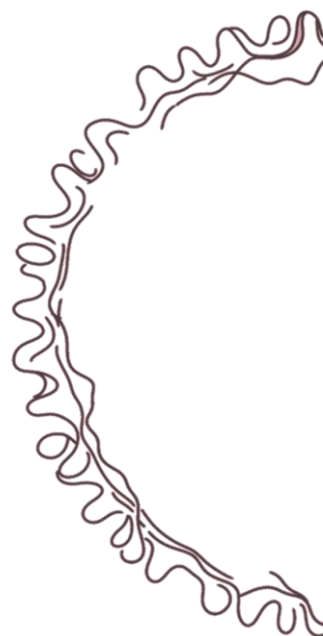
CONCLUSIONS

1. Biodistribution evaluation concluded that IV administration is the most appropriate route for clinical translation, as it enables a rapid systemic distribution and efficient targeting of key immunological organs, including the liver, spleen and secondary lymph nodes.
2. Following IV administration, PS-liposomes are taken up, cleared and processed by various immune cell subsets. Initial uptake occurs in circulating monocytes, followed by rapid accumulation in the liver, where Kupffer cells and LSECs and DCs contribute significantly to liposome internalization and processing. In the spleen PS-liposomes are captured by resident-tissue M ϕ and monocyte derived M ϕ and multiple DC subsets.
3. Administration of antigen-loaded PS-liposomes induces an antigen-specific immunoregulatory response, characterised by an increase of antigen-specific conventional and non-conventional T regulatory and B regulatory subsets after 4 days of treatment. The increase of antigen-specific anergic and exhausted T cells observed after 14 days indicate the long-term tolerance inducing potential of PS-liposomes.
4. Human DCs derived from MG and RA, as well as monocyte-derived DCs from synovial fluid exhibit a tolerogenic phenotype modulation following PS-liposome uptake. Phenotypically, this is characterised by the upregulation of PS receptors, HLA-DR, chemokine receptors and tolerogenic markers such as DC-SIGN and PD-L1, along with downregulation of costimulatory molecules such as CD86 and CD54, is characteristic. Moreover, after processing PS-loaded liposomes, these DCs are capable of presenting the encapsulated antigen via HLA-DQ and HLA-DR molecules, and of impairing autologous CD4⁺ and CD8⁺ T cell proliferation, indicating an antigen-specific tolerogenic function.
5. Human circulating B cells express PS receptors on their surface and a small subset of this cells shows ability to internalise fluorescently labelled PS-liposomes. In addition, B cells that interact with NBD-PS-loaded-liposomes produce higher levels of the anti-inflammatory cytokines IL-10 and TGF- β compared to non-interacting B cells, supporting their potential role in PS-liposome-induced immune regulation.

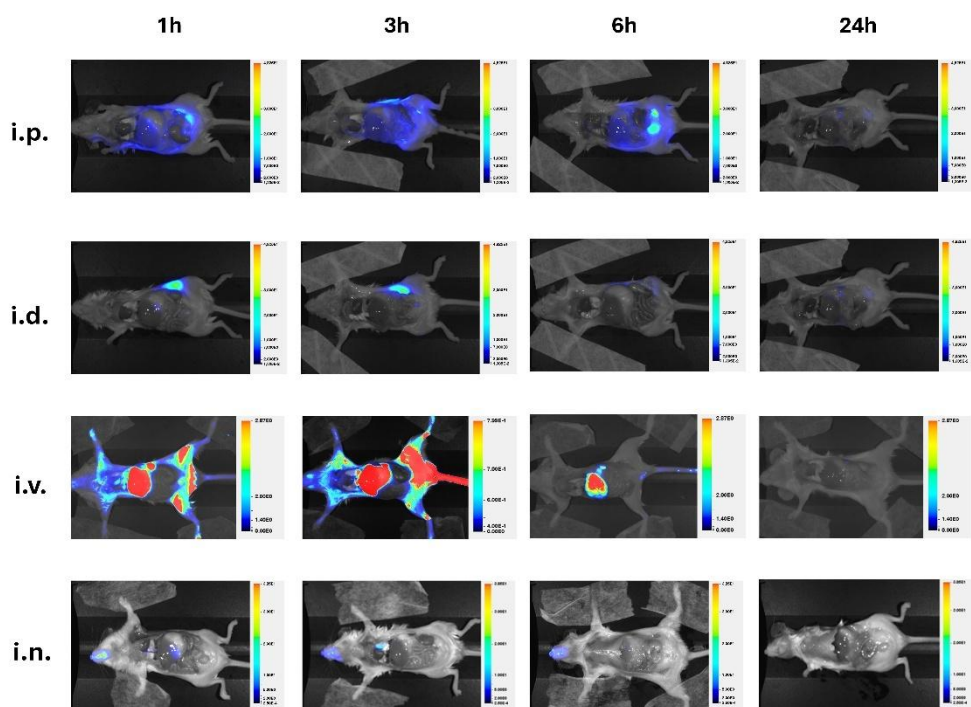
6. The hemocompatibility studies and the cytokine release assay performed support the safety and clinical translation potential of PS-AChR-liposomes, underscoring their favourable biocompatibility and non-inflammatory profile.
7. A robust and reproducible potency assay has been successfully developed, providing a valuable tool for assessing the biological activity of every PS-loaded-liposomes produced batch, and representing a critical tool for quality control in translational development.



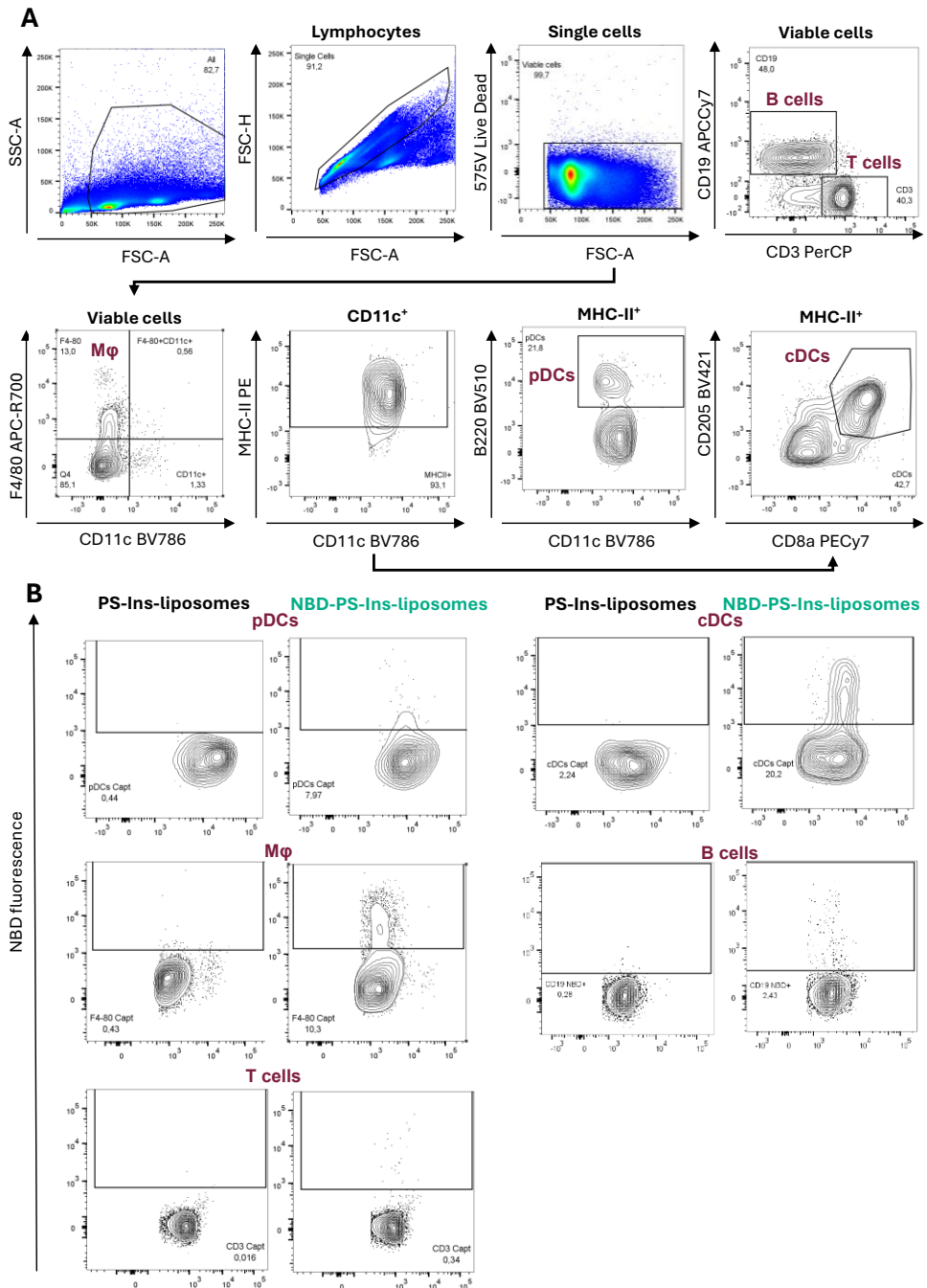
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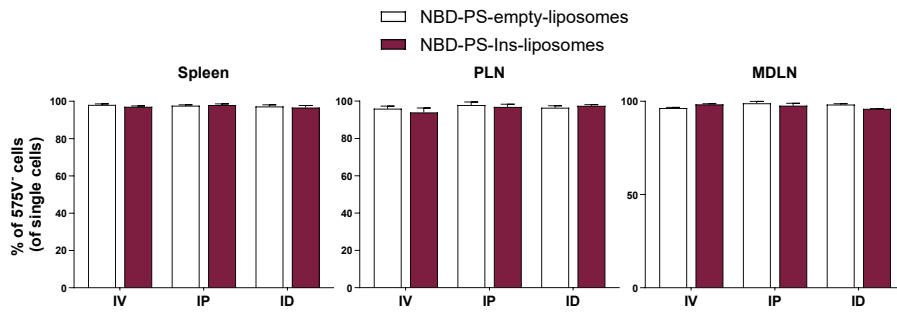
SUPPLEMENTARY DATA



Supplementary Figure 1. Fluorescence emission at 1-, 3-, 6- and 24-hour timepoint of empty DOPE-AF750-liposomes administered into prediabetic NOD female mice. Representative biodistribution images of the four different administration routes: IP (200 μ l), ID (50 μ l), IV (100 μ l) or IN (50 μ l).



Supplementary Figure 2. Flow cytometry analysis of the NBD⁺ immune cell subsets in prediabetic NOD female mice spleen after PS-Ins-liposomes or NBD-PS-Ins-liposomes administered IP, IV or ID. (A) Representative gating strategy used to analyse immune cell subsets within splenocytes. Cells were first gated for lymphocytes (FSC-A vs SSC-A), singlets (FSC-A vs FSC-H) and viable cells (575V⁻ cells). Positive cells for CD19 and CD3 were defined as B and T cells respectively. Positive cells for F4/80 were gated as macrophages (M ϕ). CD11c⁺ and MHC-II⁺ cells were selected and plasmacytoid dendritic cells (pDCs) were defined as B220 positive cells while conventional dendritic cells (cDCs) as CD205 and CD8 double positive cells. FMO for MHC-II and F4/80 were used to define the gates (not shown). **(B)** Representative contour plots of each pairwise subset of immune cells, mice injected with non-fluorescent PS-Ins liposomes (left panel) or with NBD-labelled PS-Ins liposomes (right panel).



Supplementary Figure 3. Percentage of viable cells from prediabetic NOD female mice spleen, PLN and MDLN after NBD-labelled empty PS-liposomes or PS-Ins-liposome IP, IV or ID. Data represented as mean $\pm$ SEM of 575V⁺ cells from single cells.

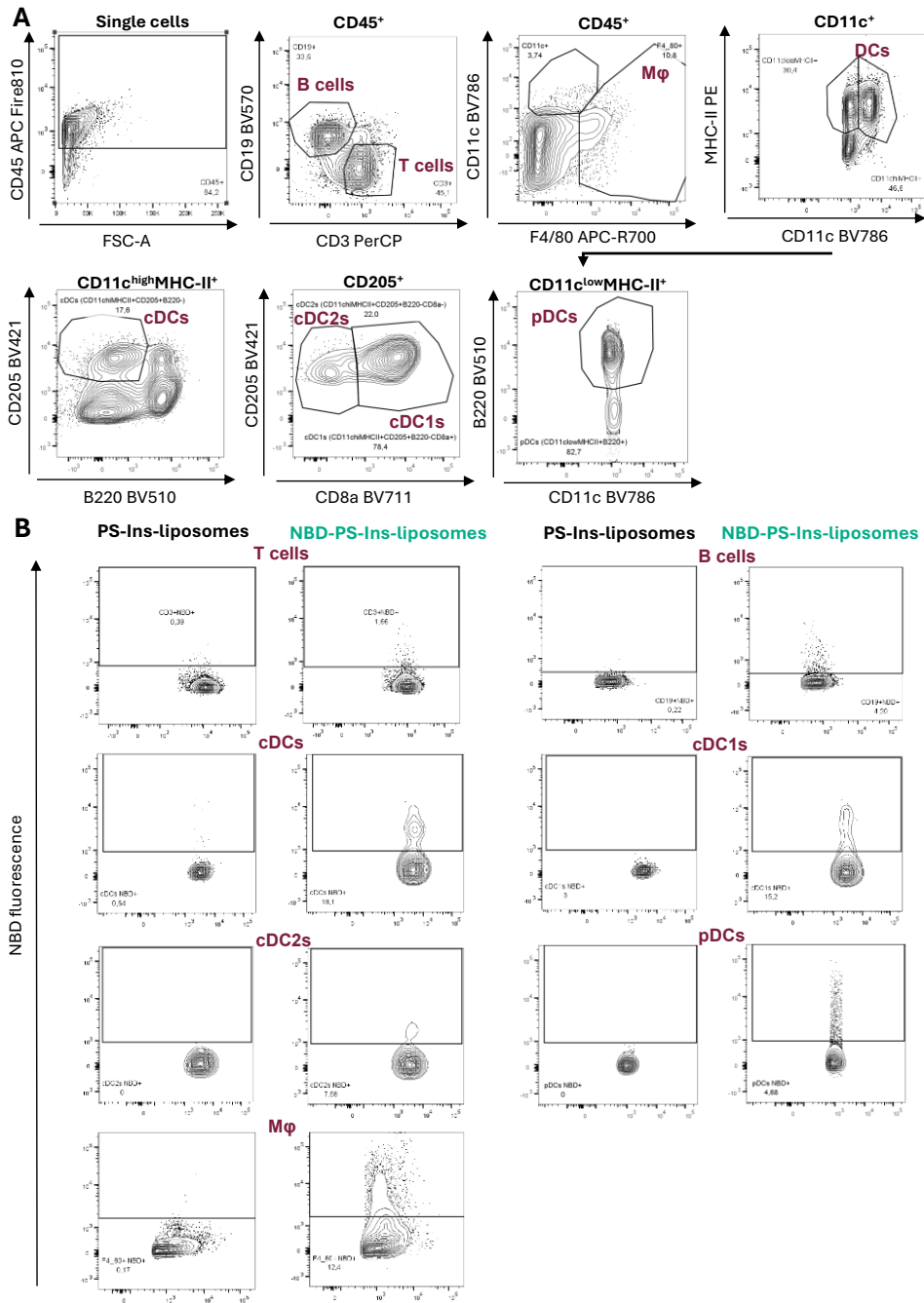
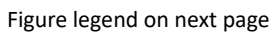
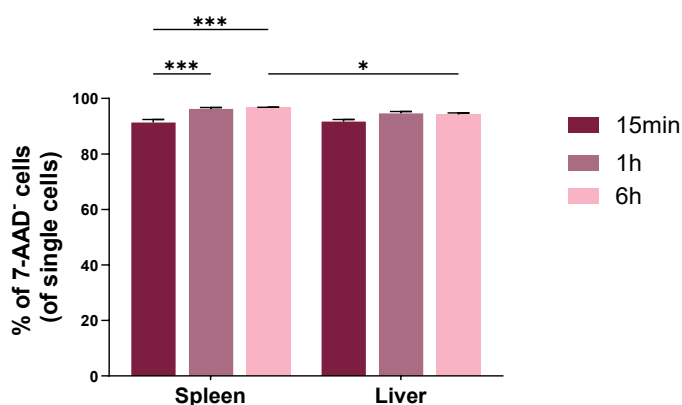


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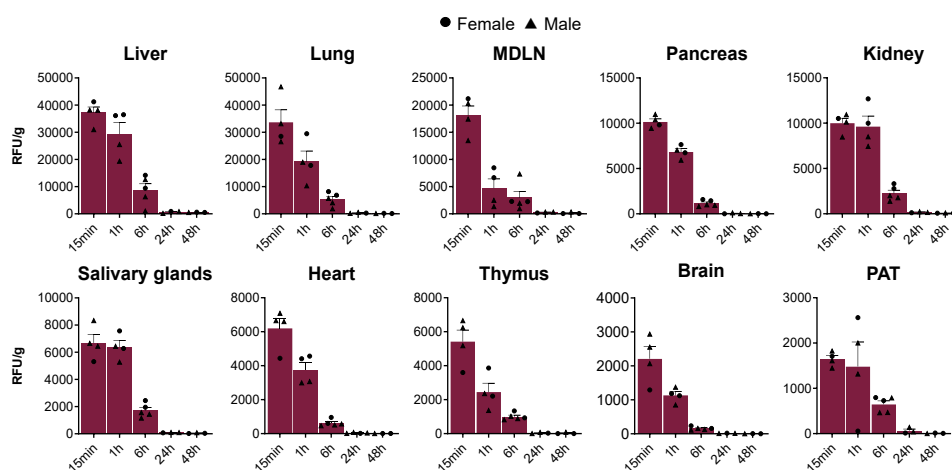
Supplementary Figure 4. Flow cytometry strategy for the NBD⁺ immune cell subsets in NOD mice spleen after IV administration of PS-Ins-liposomes. (A) Representative gating strategy used to analyse immune cell subsets within CD45⁺ splenocytes. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H) (not shown). Positive cells for CD19 and CD3 were defined as B and T cells respectively. Positive cells for F4/80 were gated as Mφ. CD11c⁺ cells were selected and plasmacytoid pDCs were defined as B220 positive and MHC-II low expression cells while cDCs as CD205 positive and MHC-II high expression cells. Conventional DCs type 1 (cDC1s) were selected as CD8 negative cells, whereas, conventional DCs type 2 (cDC2s), as CD8 positive cells. FMO for CD45, MHC-II and F4/80 were used to define the gates (not shown). **(B)** Representative contour plots of each pairwise subset of immune cells, mice injected with non-fluorescent PS-Ins liposomes (left panel) or with NBD-labelled PS-Ins liposomes (right panel).



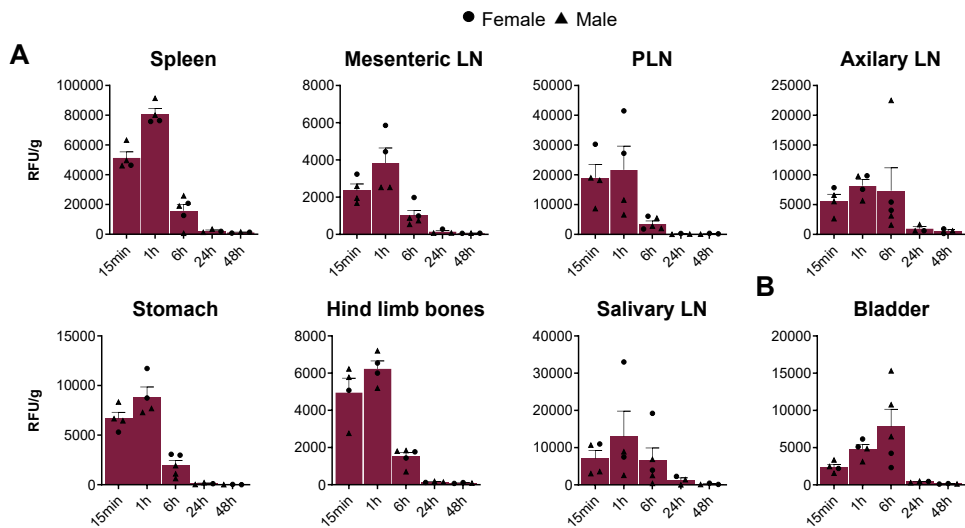
Supplementary Figure 5. Flow cytometry strategy for the NBD⁺ immune cell subsets in NOD mice liver after IV administration of PS-Ins-liposomes. Representative gating strategy used to analyse immune cell subsets within liver cells. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H) (not shown). CD45⁺ cells were gated and CD206 positive cells were selected as liver sinusoidal endothelial cells (LSECs). Regarding CD45⁺ cells, positive cells for CD19 and CD3 were defined as B and T cells respectively (not shown). Positive cells for F4/80 were gated and double CD68 and MHC-II positive cells were categorised as Kupffer cells while CD68 negative cells but CD14 expressing cells were gated as monocyte derived M ϕ . CD11c⁺ cells were selected and plasmacytoid pDCs were defined as B220 positive and MHC-II low expression cells while cDCs as CD205 positive and MHC-II high expression cells (not shown). Conventional DCs type 1 (cDC1s) were selected as CD8 negative cells, whereas, conventional DCs type 2 (cDC2s), as CD8 positive cells (not shown). FMO for CD45, MHC-II and F4/80 were used to define the gates (not shown). **(B)** Representative contour plots of each pairwise subset of immune cells, mice injected with non-fluorescent PS-Ins liposomes (left panel) or with NBD-labelled PS-Ins liposomes (right panel).



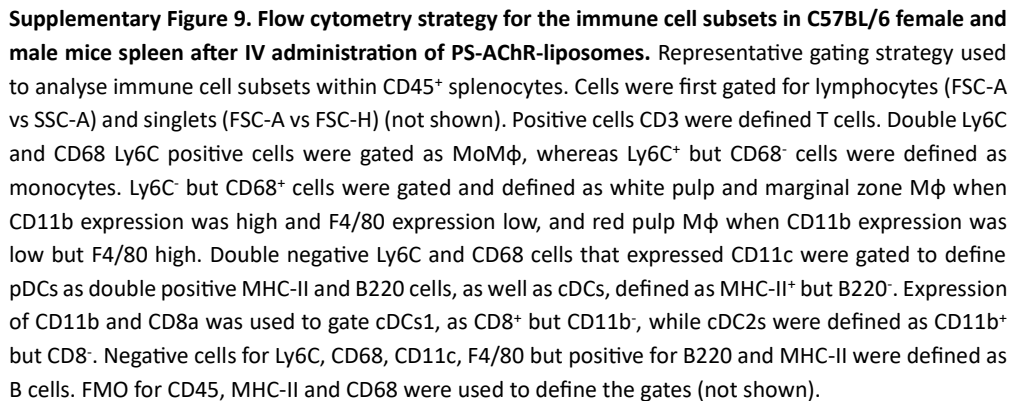
Supplementary Figure 6. Percentage of viable cells of prediabetic NOD female mice spleen and liver after 15 minutes, 1 hour and 6 hours after PS-Ins-liposome IV administration. Data represented as mean $\pm$ SEM of percentage of viable cells 7-AAD⁻ cells from single cells studied by flow cytometry.

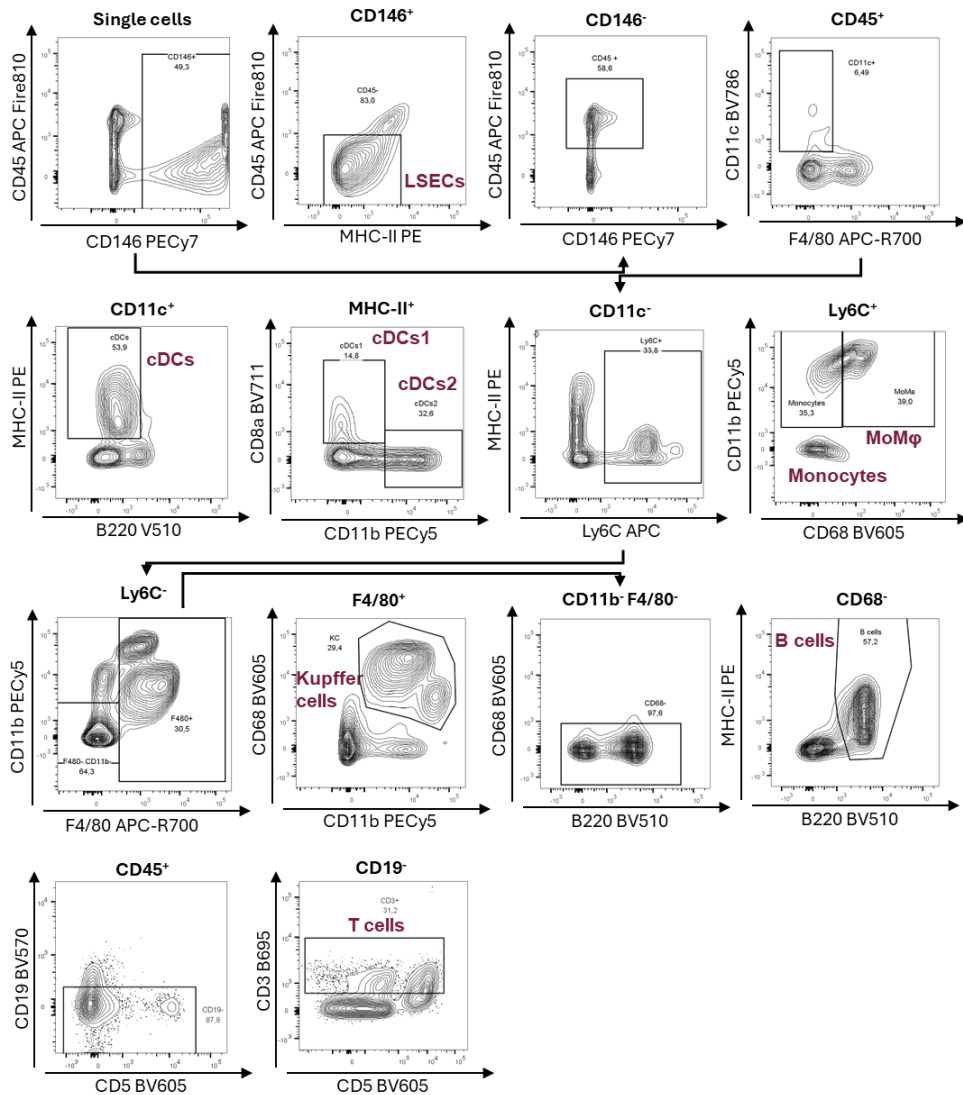


Supplementary Figure 7. Mice organs that presented the fluorescent DOPE-AF750-labelled PS-AChR-liposomes peak after 15 minutes post IV administration (n=3-6). The rounded dots represent females, the triangles represent males. Data represented as mean $\pm$ SEM of relative fluorescent signal (RFU/g of tissue), assessed by fluorescent imaging.

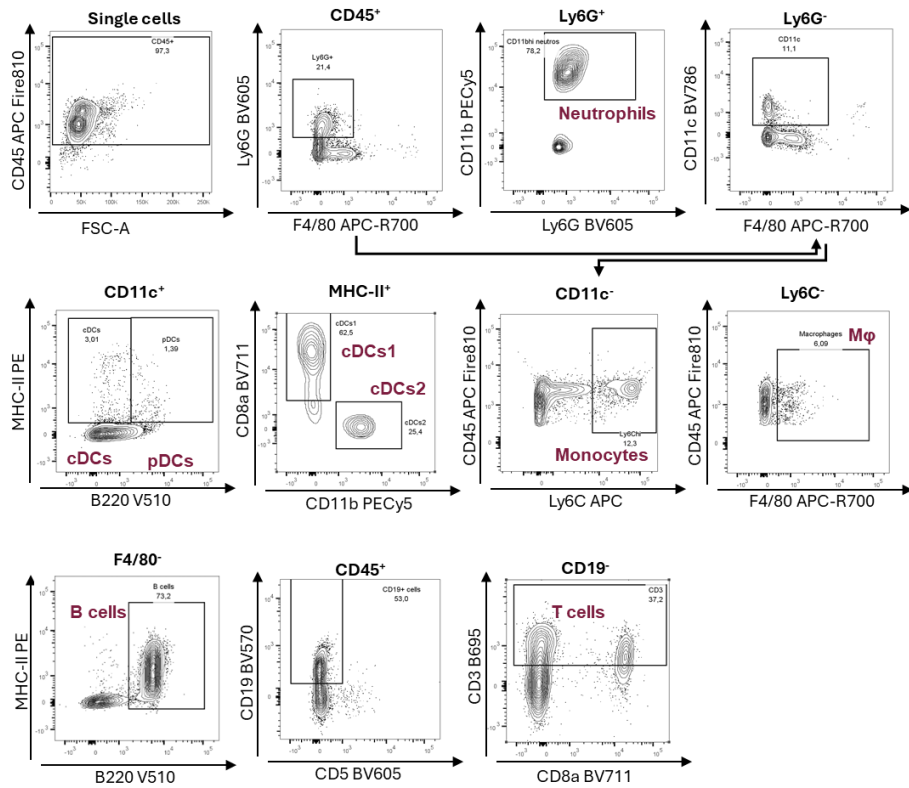


Supplementary Figure 8. Mice organs that fluorescent DOPE-AF750-labelled PS-AChR-liposomes peak after 1h post IV administration (n=3-6). (A) Organs that present fluorescence peak at 1h after administration. **(B)** Organ that present fluorescence peak after 6h of administration. The rounded dots represent females; the triangles represent males. Data represented as mean $\pm$ SEM of relative fluorescent signal (RFU/g of tissue), assessed by fluorescent imaging.

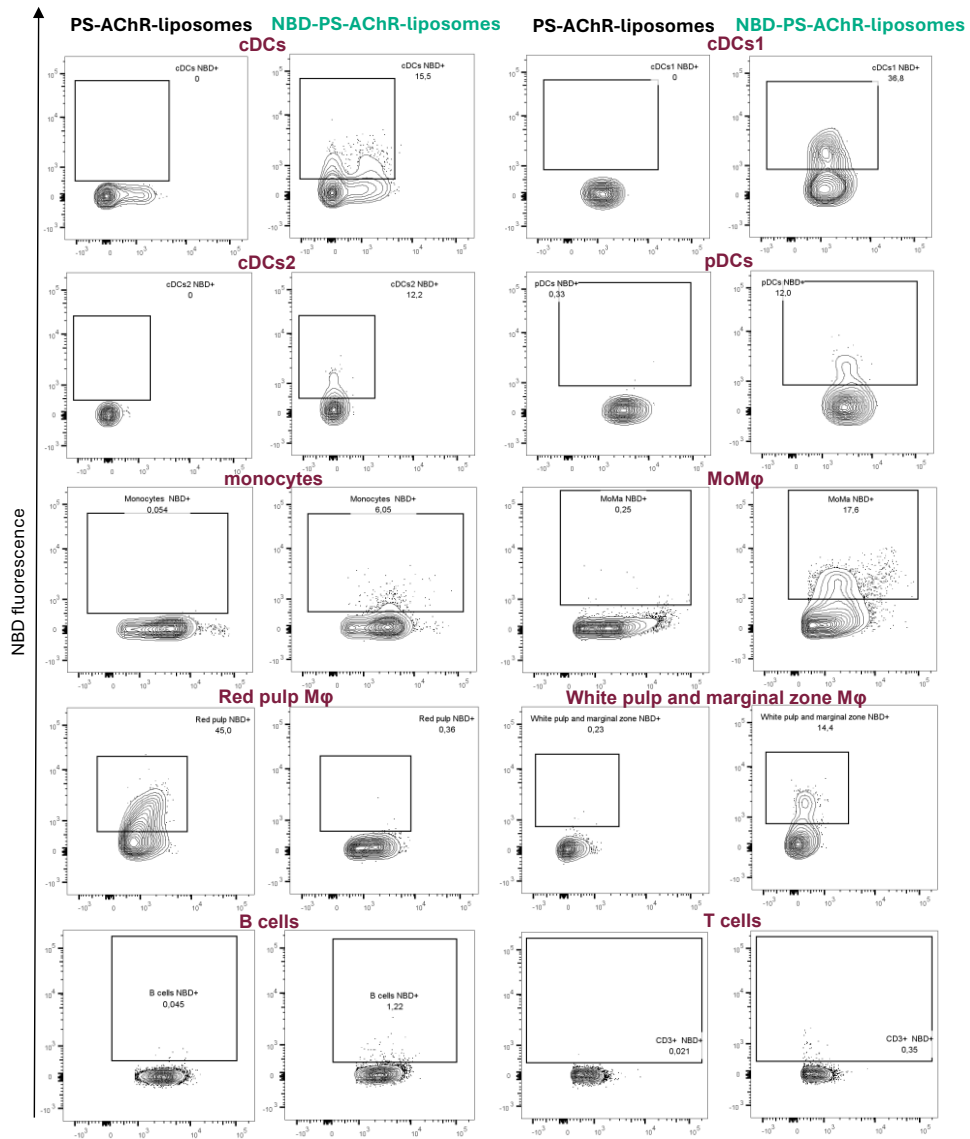




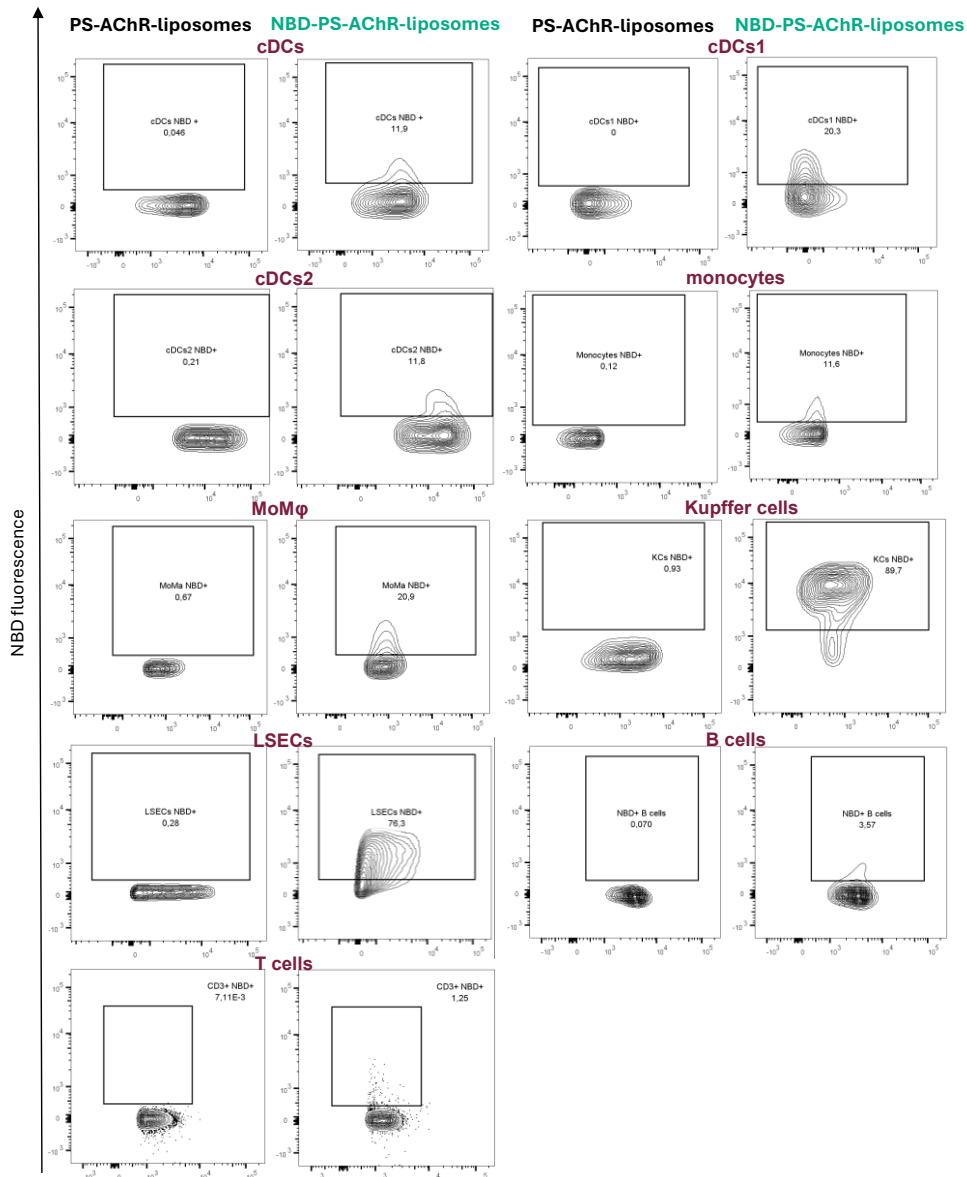
Supplementary Figure 10. Flow cytometry strategy for the immune cell subsets in C57BL/6 female and male mice liver after IV administration of PS-AChR-liposomes. Representative gating strategy used to analyse immune cell subsets within liver cells. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H) (not shown). CD146⁺ cells were gated and CD45 negative cells were selected as liver LSECs. Regarding CD45⁺ cells, positive cells CD3 were defined T cells. CD11c positive cells were gated to define pDCs as double positive MHC-II and B220 cells (not shown), as well as cDCs, defined as MHC-II⁺ but B220⁻. Expression of CD11b and CD8a was used to gate cDCs1, as CD8⁺ but CD11b⁻, while cDC2s were defined as CD11b⁺ but CD8⁻. CD11c⁻ cells but Ly6C⁺ were selected and CD68⁻ cell were defined as monocytes whereas CD68⁺ cells were defined as MoMφ. Then Ly6C negative cells were gated to select double F4/80 and CD68 positive cells defining Kupffer cells. Double negative CD11b and F4/80 cells were then gated to select B220 positive cells defined as B cells. FMO for CD45, MHC-II and CD68 were used to define the gates (not shown).



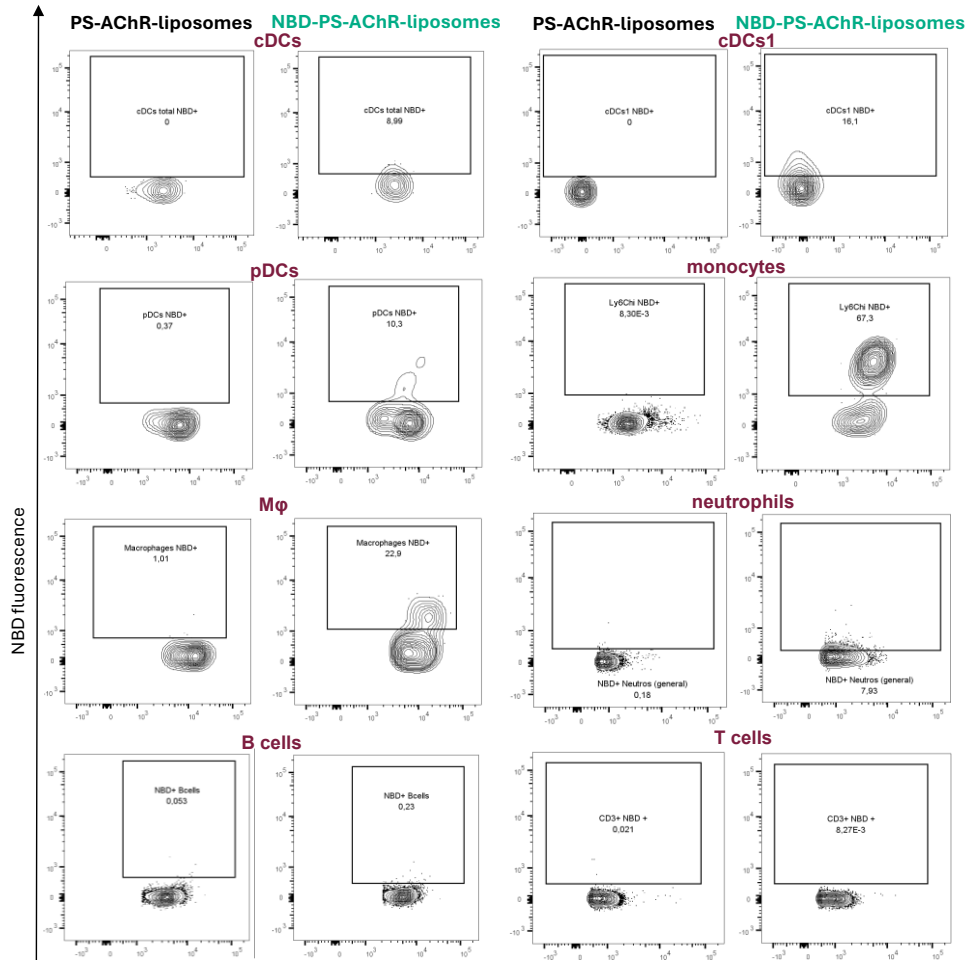
Supplementary Figure 11. Flow cytometry strategy for the immune cell subsets in C57BL/6 female and male mice blood after IV administration of PS-AChR-liposomes. Representative gating strategy used to analyse immune cell subsets within CD45⁺ cells. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H) (not shown). Positive cells CD3 were defined T cells. Double positive Ly6G and CD11b cells were defined as neutrophils. Ly6G⁻ cells were gated to select CD11c positive cells and define pDCs as MHC-II and B220 positive cells. cDCs, defined as MHC-II⁺ but B220⁻, were differentiated into cDCs1, as CD8⁺ but CD11b⁻ and cDCs2, defined as CD11b⁺ but CD8⁻. CD11c⁻ but Ly6C⁺ cells were gated as monocytes. Ly6C⁻ but F4/80 cells were defined as Mφ. Negative cells for CD11c, Ly6C, F4/80 but positive for B220 and MHC-II were defined as B cells. FMO for CD45, MHC-II and CD68 were used to define the gates (not shown).



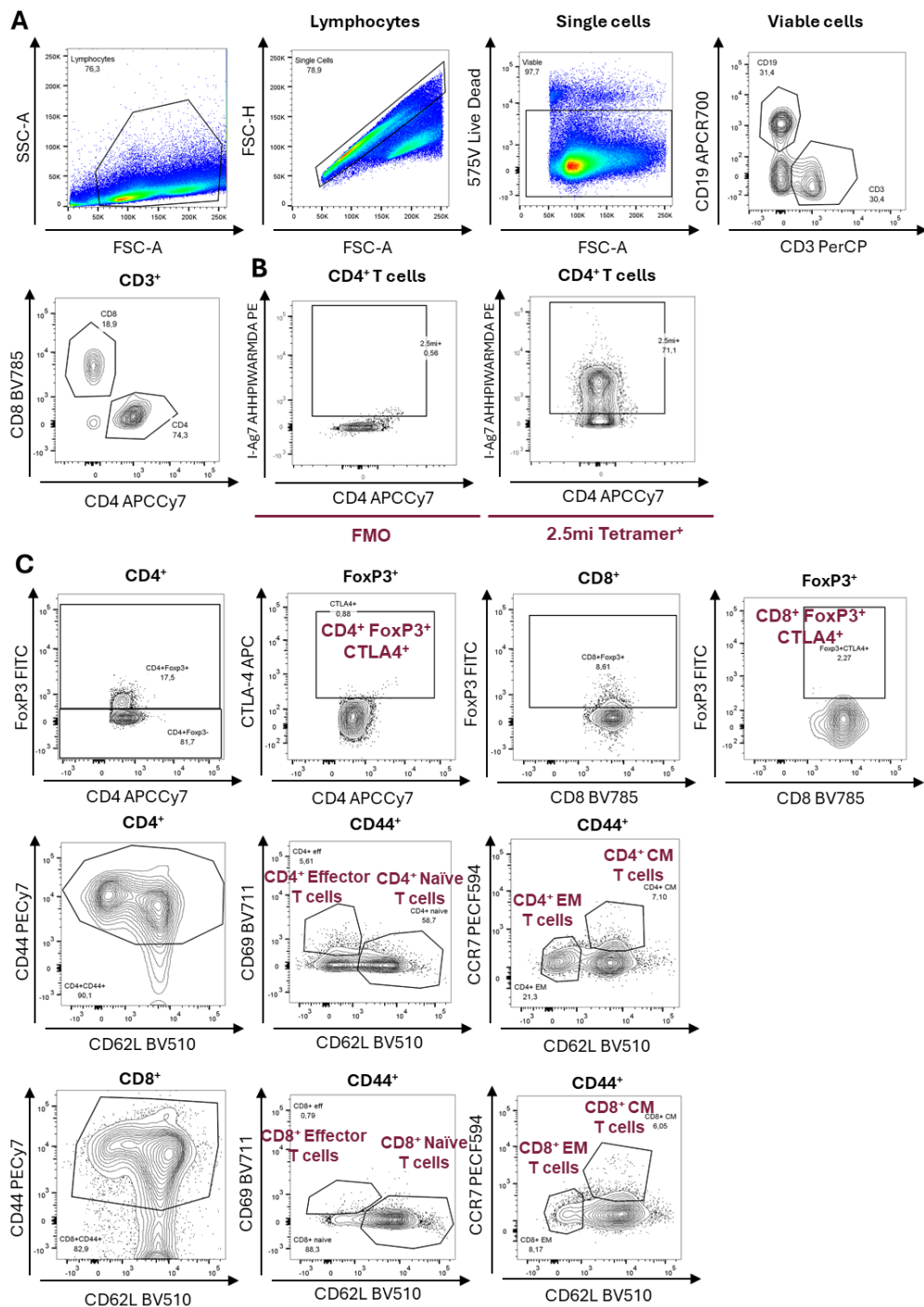
Supplementary Figure 12. Gating representing immune cell subsets in spleen from C57BL/6 female and male mice injected with non-fluorescent PS-AChR-liposomes or NBD-labelled PS-AChR-liposomes. Representative contour plots of each pairwise subset of immune cells, mice injected with non-fluorescent PS-AChR-liposomes (left panel) or with NBD-labelled PS-AChR-liposomes (right panel).

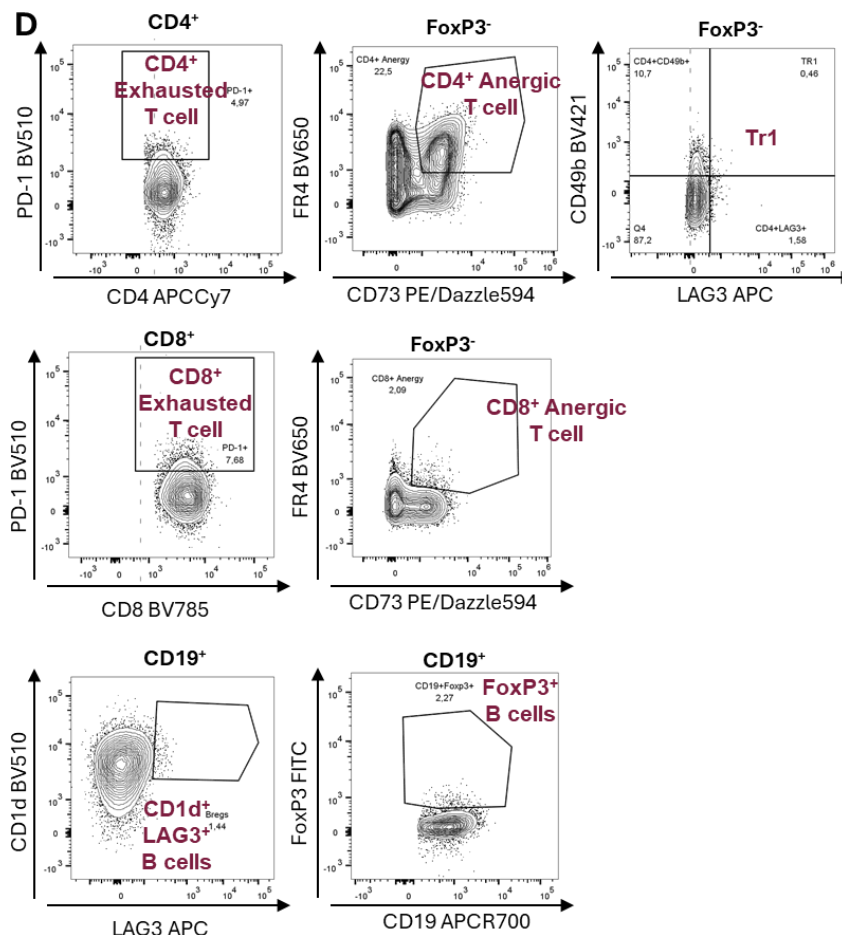


Supplementary Figure 13. Gating representing immune cell subsets in liver from C57BL/6 female and male mice injected with non-fluorescent PS-AChR-liposomes or NBD-labelled PS-AChR-liposomes. Representative contour plots of each pairwise subset of immune cells, mice injected with non-fluorescent PS-AChR-liposomes (left panel) or with NBD-labelled PS-AChR-liposomes (right panel).



Supplementary Figure 14. Gating representing immune cell subsets in blood from C57BL/6 female and male mice injected with non-fluorescent PS-AChR-liposomes or NBD-labelled PS-AChR-liposomes. Representative contour plots of each pairwise subset of immune cells, mice injected with non-fluorescent PS-AChR-liposomes (left panel) or with NBD-labelled PS-AChR-liposomes (right panel).





Supplementary Figure 15. Flow cytometry strategy for analysing the immune cell subsets in BDC2.5mi mice spleen after IV or ID administration of PS-2.5mi-liposomes. (A) Representative gating strategy used to analyse immune cell subsets within splenocytes. Cells were first gated for lymphocytes (FSC-A vs SSC-A), singlets (FSC-A vs FSC-H) and viable cells (575V⁻ cells). Positive cells for CD19 or CD3 were defined as B or T cells, respectively. Then CD3⁺ cells were gated to select CD4⁺ or CD8⁺ T cells. **(B)** Representative contour plot of CD4⁺ T cells expressing 2.5mi TCR, FMO (left panel) and 2.5mi tetramer (right panel). **(C)** Both CD4⁺ and CD8⁺ T cells were gated to select double FoxP3 and CTLA-4 T cells, defined as conventional Tregs. CD44 positive cells were gated to define CD69⁺ but CD62L⁻ as effector T cells while CD69⁻ but CD62L⁺ were defined as naïve T cells. Double positive CCR7 and CD62L cells were defined as central memory (CM) T cells and double negative cells, as effector memory (EM) T cells. **(D)** Both CD4⁺ and CD8⁺ T cells were gated to select PD-1 cells, defined as exhausted T cells, whereas double CD73 and FR4 cells were defined as anergic T cells. CD4⁺ T cells expressing CD49b and LAG3 were gated as non-conventional Tr1 cells. CD19⁺ cells were gated to select CD1d⁺ and LAG3⁺, and FoxP3⁺ B regulatory cells. FMOs of CD25, CTLA-4, CD44, CD62L, CD69, CCR7, CD49b, LAG3, CD73, FR4, PD-1, TIGIT, CD1d, MHC-II were used to define the gates (not shown).

Supplementary Table 1. Clinical features of MG patients used in SECTION III of this study

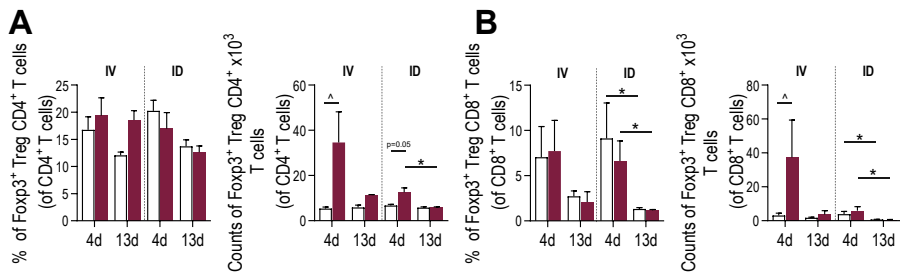
Patient	Anti-AChR (nmol/L)	Clinical manifestation	Thymic pathology	MGFA score (at the day of extraction)	HLA-DR	HLA-DQ
MG01	125	Generalised	No	IIIA	-	-
MG02	54	Generalised	No	I	-	-
MG03	129	Generalised	Hyperplasia	IIA	DRB1*01 DRB1*01	DQA1*01 DQA1*01 DQB1*05 DQB1*05
MG04	107	Generalised	No	IIIB	DRB1*01, DRB1*04	DQA1*01, DQA1*03 DQB1*03, DQB1*05
MG05	6	Ocular	No	I	-	-
MG06	106	Generalised	No	IIIB	-	-
MG07	32	Ocular	No	I	DRB1*01, DRB1*09	DQA1*01, DQA1*03 DQB1*03, DQB1*05
MG08	6	Generalised	No	IIIB	-	-
MG09	17	Generalised	No	IIIA	DRB1*01, DRB1*07	DQA1*01, DQA1*02 DQB1*02, DQB1*05
MG10	67	Generalised	No	IVB	-	-
MG11	29	Ocular	No	I	-	-

AChR: acetyl choline receptor; MG: Myasthenia gravis; MGFA: myasthenia gravis foundation of America

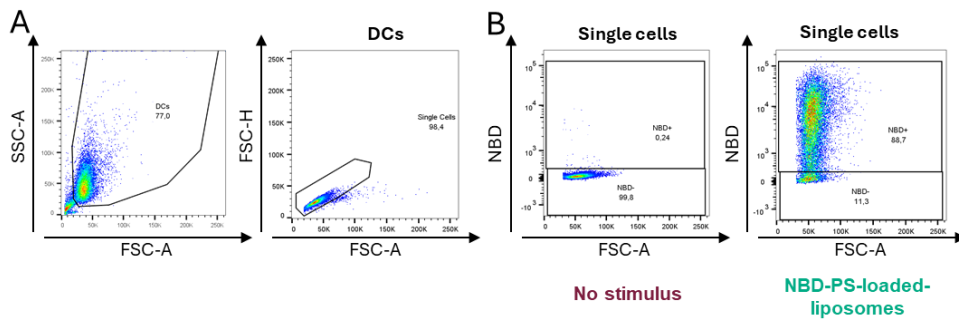
Supplementary Table 2. Clinical features of RA patients used in SECTION III of this study

Patient	RF	ACPA	DAS28	HLA DR
RA01	Positive	Positive	4,66	DRB1*09, DRB1*15
RA02	Positive	Positive	6,33	DRB1*04, DRB1*09
RA03	Positive	Positive	4,78	DRB1*09:01P DRB1*11:01P
RA04	Positive	Positive	2,9	DRB1*01 DRB1*03
RA05	Positive	Positive	6,37	DRB1*01:01P DRB1*16:01P
RA06	Positive	Positive	4,85	DRB1*04, DRB1*16
RA07	Positive	Positive	3,14	DRB1*09:01 DRB1*11:01
RA08	Positive	Positive	5,2	-
RA09	Negative	Negative	3,1	DRB1*04:01P DRB1*04:01P
RA10	Positive	Negative	5,46	DRB1*1, DRB1*04, DRB1*01:01P, DRB1*01:02P, DRB1*04:01P
RA11	Positive	Positive	4,05	-
RA12	Negative	Negative	5,4	-

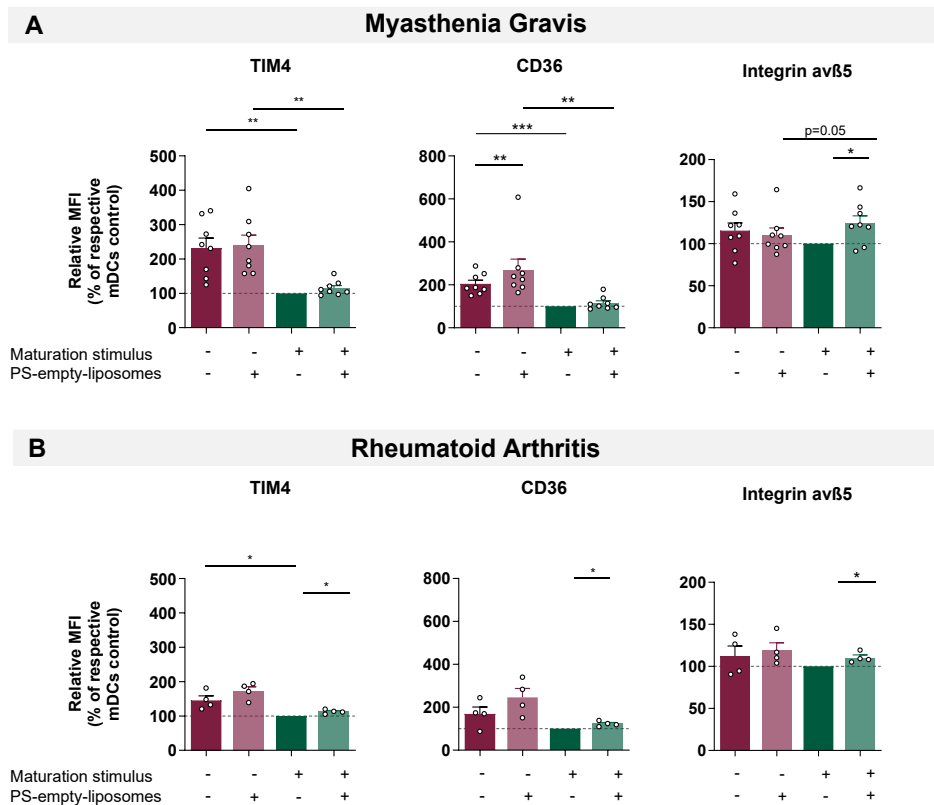
ACPA: anti-citrullinated peptide antibodies; DAS28: disease activity score-28; RF: rheumatoid factor



Supplementary Figure 16. Percentages and cell count of FoxP3⁺ T cells from the spleen of NOD.BDC2.5 mice after 4 or 13 days of the IV or ID PS-liposomes or PS.2.5mi-liposomes administration (n=4). (A) Percentages and total cell count of CD4⁺ T cell compartment and **(B)** CD8⁺ T cell compartment. Bars represent PS-empty-liposomes (white) and PS-2.5mi-liposomes (dark pink). Data represented as mean ± SEM. Significant differences were found when comparing the groups (^p < 0.05 between liposome treatment, *p < 0.05 between timepoints, Mann-Whitney test). From Almenara-Fuentes et al., 2023.

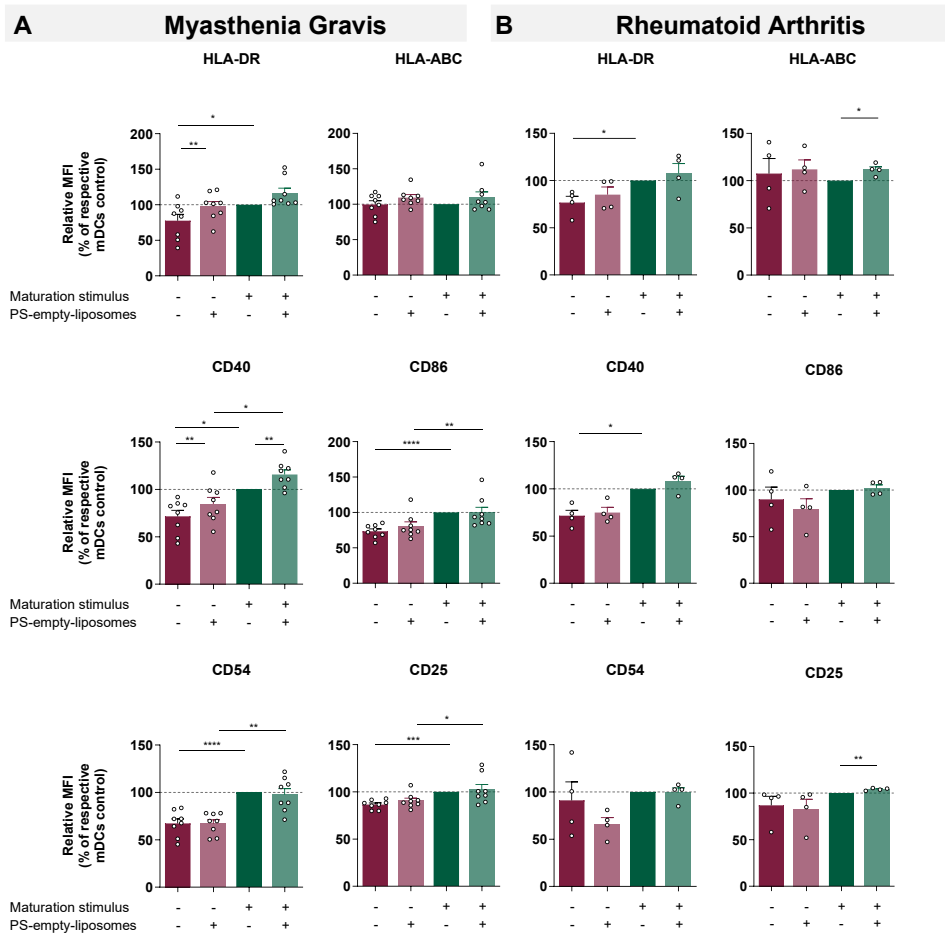


Supplementary Figure 17. Flow cytometry analysis of the monocyte derived DCs from MG and RA patients. (A) Gating strategy used to analyse phenotype markers of DCs after their differentiation. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H) **(B)** Gating strategy used to analyse the percentages of NBD⁺ DCs after engulfing NBD-labelled PS-loaded-liposomes (right panel) vs conditions with no stimulus (left panel).

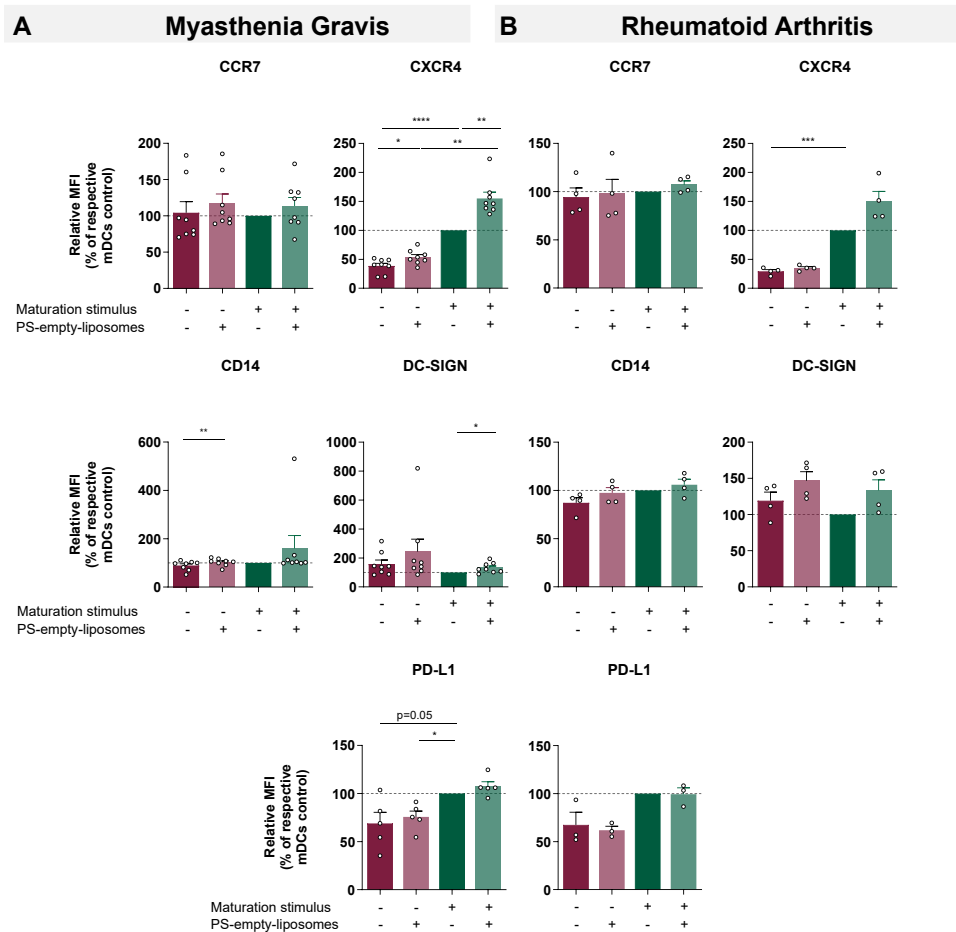


Supplementary Figure 18. PS receptors are modulated in DCs after PS-empty-liposomes phagocytosis.

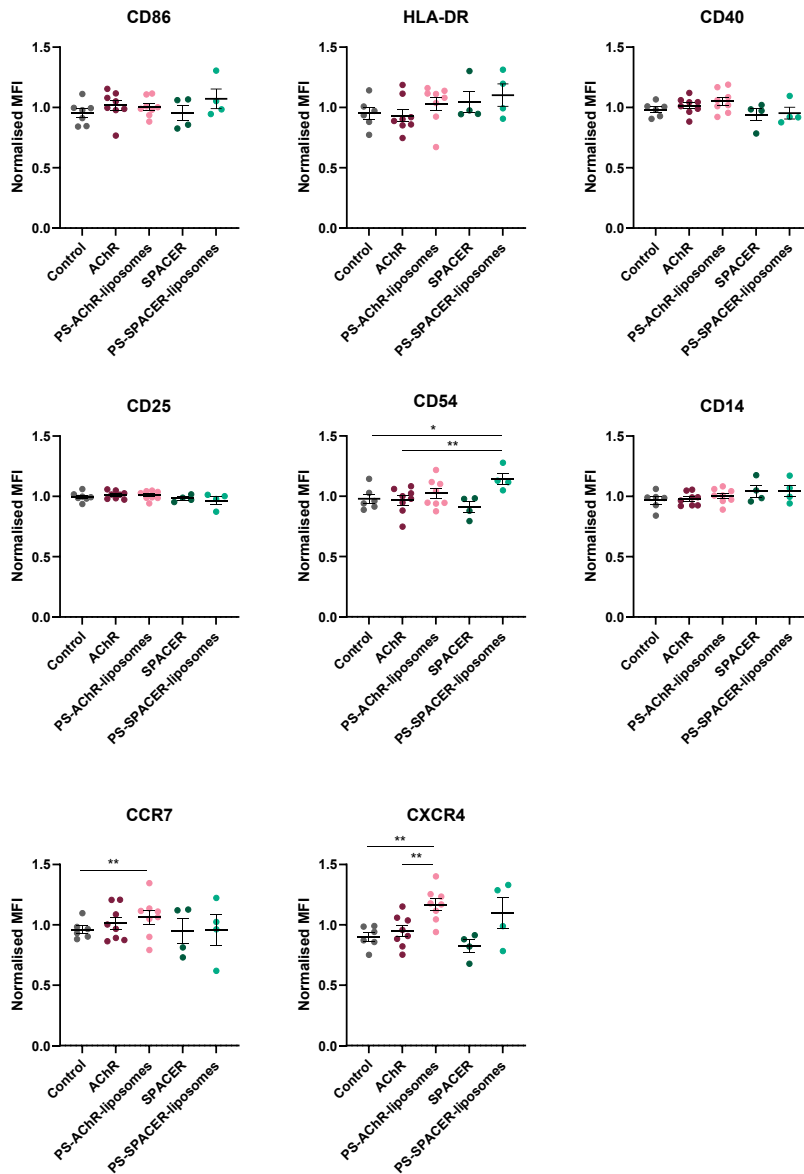
(A) Relative MFI levels of TIM-4, CD36 and Integrin $\alpha v \beta 5$ membrane expression on iDCs and mDCs obtained from MG ($n \geq 8$) and **(B)** RA patients ($n \geq 4$). Bars represent iDCs (dark pink), iDCs after PS-empty-liposome phagocytosis (purple), mDCs (dark green) or mDCs after PS-empty-liposome phagocytosis (greyish-green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions (** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition (** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, One sample Wilcoxon signed-rank test).



Supplementary Figure 19. PS-empty-liposomes phagocytosis modulates immune phenotype markers in DCs. (A) Relative MFI values of HLA-DR, HLA-ABC, CD40, CD86, CD54 and CD25 membrane expression on iDCs and mDCs obtained from MG ($n \geq 8$) and **(B)** RA ($n = 4$). Bars represent iDCs (dark pink), iDCs after PS-empty-liposome phagocytosis (purple), mDCs (dark green) or mDCs after PS-empty-liposome phagocytosis (greyish-green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions ($***p < 0.001$, $**p < 0.01$, $*p \leq 0.05$, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition ($***p < 0.0001$, $**p < 0.001$, $**p < 0.01$, $*p \leq 0.05$, One sample Wilcoxon signed-rank test).



Supplementary Figure 20. PS-empty-liposomes phagocytosis modulates immune phenotype markers in DCs. (A) Relative MFI values of CCR7, CXCR4, CD14, DC-SIGN and PD-L1 membrane expression on iDCs and mDCs obtained from MG ($n \geq 8$) and **(B)** RA patients ($n \geq 4$). Bars represent iDCs (dark pink), iDCs after PS-empty-liposome phagocytosis (purple), mDCs (dark green) or mDCs after PS-empty-liposome phagocytosis (greyish-green). Data represented as mean $\pm$ SEM of relative MFI, with the MFI of each culture condition normalized to their respective mDCs control. Significant differences were found when comparing among culture conditions (*** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test) and when comparing a culture condition with the mDCs control condition (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, One sample Wilcoxon signed-rank test).



Supplementary Figure 21. PS-loaded-liposomes phagocytosis modulates immune phenotype markers in mDCs from control subjects. Dots represent control condition (grey), mDCs cultured with AChR peptide (dark pink), mDCs cultured with PS-AChR-liposomes (light pink), mDCs cultured with SPACER peptide (dark green) and mDCs cultured with PS-SPACER-liposomes (light green). Data represented as mean $\pm$ SEM and normalised MFI, by dividing the value of each data point by the value of the pooled mean of the respective experiment. Significant differences were found when comparing among culture conditions (***) $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, Wilcoxon matched-pairs signed rank test).

Supplementary Table 3. HLA class II haplotypes of healthy controls used for the proteomic study

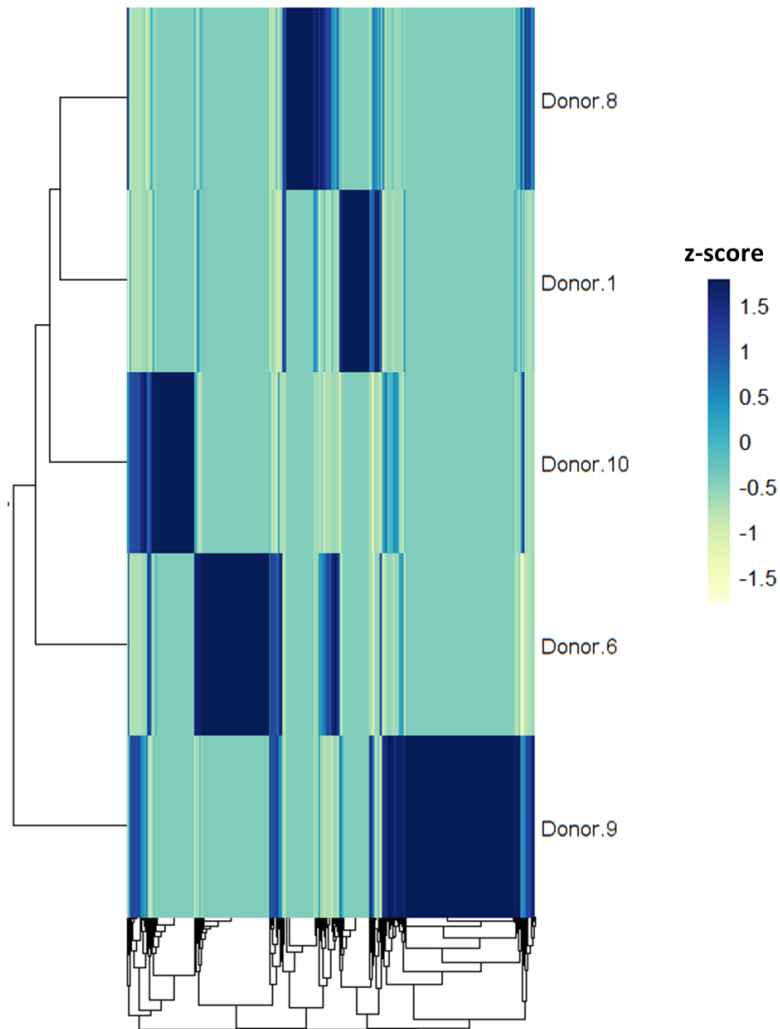
D	HLA-DRB1		HLA-DR3		HLA-DR4		HLA-DRA		HLA-DQA1		HLA-DQB1	
1	11:04 P	13:02: 01	P	P	-	-	01:01: 01:05	01:01: 01:07	01:02: 01:14	05:0 5:01	03:01 P	06:04 P
2	11:01 P	15:01 P	P	-	-	-	01:01: 01:07	01:02: 03:01	01:02: 01	05:0 5:01	03:01 P	06:02 P
4	07:01 P	13:01 P	P	-	-	-	01:01: 01:02	01:02: 02:08	01:03: 01	02:0 1:01	02:01 P	06:03 P
5	01:01 P	13:01 P	P	-	P	-	01:01: 01:03	01:01: 01:05	01:01: 01	01:0 3:01	05:01 P	06:03 P
6	03:01 P	03:01 P	P	P	-	-	01:02: 02:01	01:02: 02:01	05:01: 01	05:0 1:01	02:01 P	02:01 P
7	03:01 P	03:01 P	P	P	-	-	01:02: 02:01	01:02: 02:01	05:01: 01	05:0 1:01	02:01 P	02:01 P
8	11:01 P	13:01 P	P	P	-	-	01:01: 01:07	01:02: 02:08	01:10	05:0 5:01	03:01 P	06:03 P
9	04:04 P	11:01 P	P	-	P	-	01:01: 01:03	01:01: 01:07	03:01: 01:01	05:0 5:01	03:01 P	03:02 P
10	01:01: 01	12:JV	NP	NP	NP	NP	NP	NP	NP	NP	05:01	03:01: 01

D: donor; NP: not performed; P: present

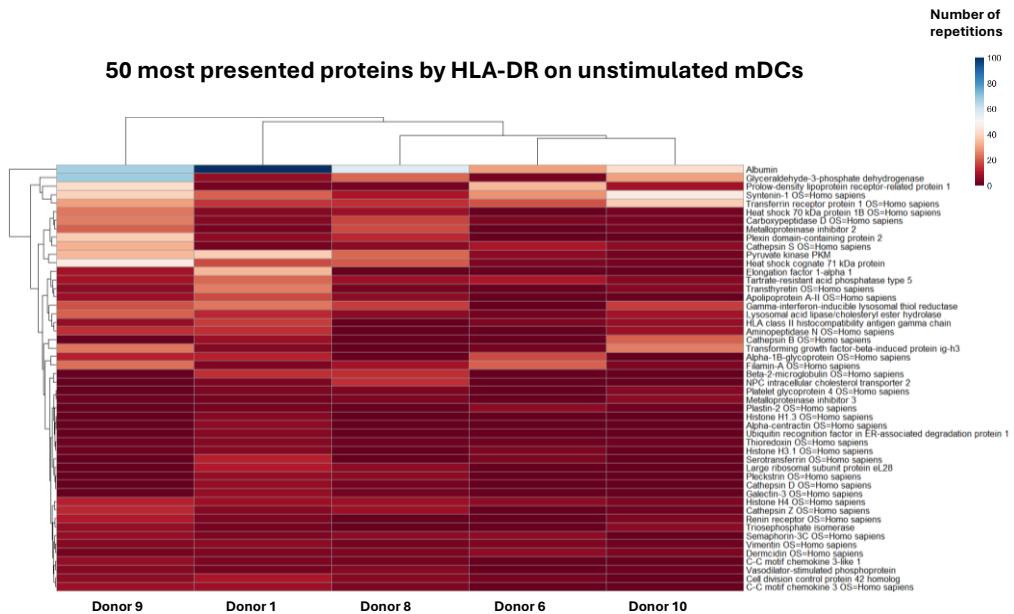
Supplementary Table 4. Summary of the number peptides presented by HLA class II molecules identified in mDCs basal conditions.

HLA-DR			HLA-DQ		
Donor	Nº of proteins	Nº of peptides	Donor	Nº of proteins	Nº of peptides
1	207	816	D1	23	102
6	336	1273	D4	20	27
8	286	928	D5	148	614
9	468	2393	D6	96	290
10	277	1219	D10	27	42

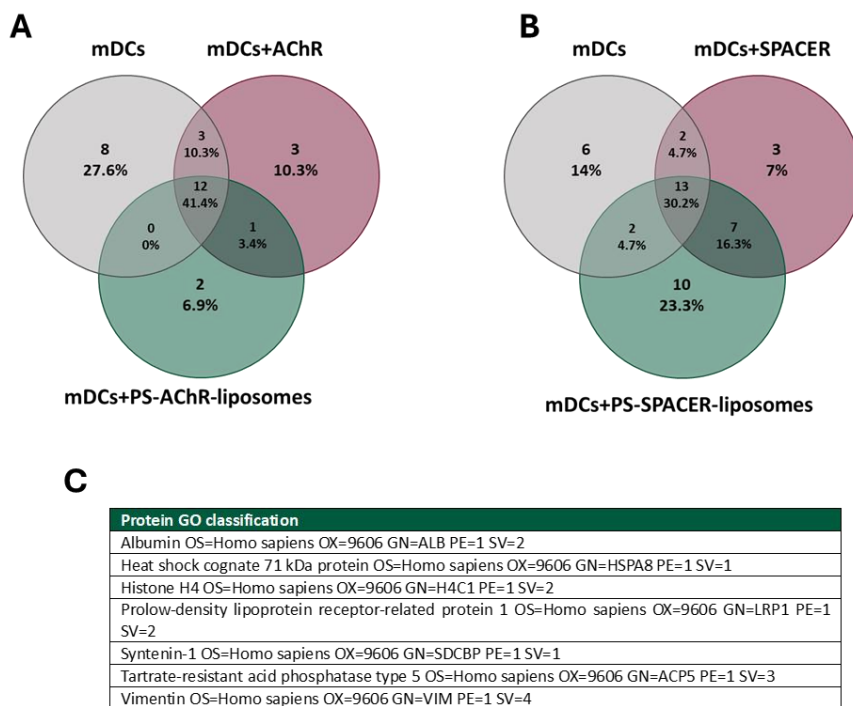
Proteins presented by HLA-DR on unstimulated mDCs



Supplementary Figure 22. Differences between the immunopeptidome presented by HLA-DR on basal mDCs conditions. Hierarchical clustering heatmap showing clusters of different proteins, colour gradient ranging from blue to white to red represent low, intermediate, and high abundance levels, respectively. Displayed values within the heatmap correspond to z-score normalised values.



Supplementary Figure 23. Representation of the 50 most presented peptides by HLA-DR of the proteins on basal mDCs conditions. Hierarchical clustering heatmap showing clusters of different peptides, colour gradient ranging from blue to white to red was defined representing low, intermediate, and high abundance levels, respectively. Displayed values within the heatmap correspond to raw count values.



Supplementary Figure 24. Common proteins which peptides were presented by HLA-DR among all mDCs conditions. (A) Venn diagram showing counts and percentages of overlapping proteins between unstimulated, AChR peptide and PS-AChR-liposomes conditions. **(B)** Venn diagram showing counts and percentages of overlapping proteins between unstimulated, SPACER peptide and PS-SPACER-liposomes conditions. **(C)** Gene ontology (GO) list of the 7 proteins shared in all matured DCs conditions.

Supplementary Table 5. Summary of the number peptides presented by HLA class II molecules identified in AChR peptide conditions

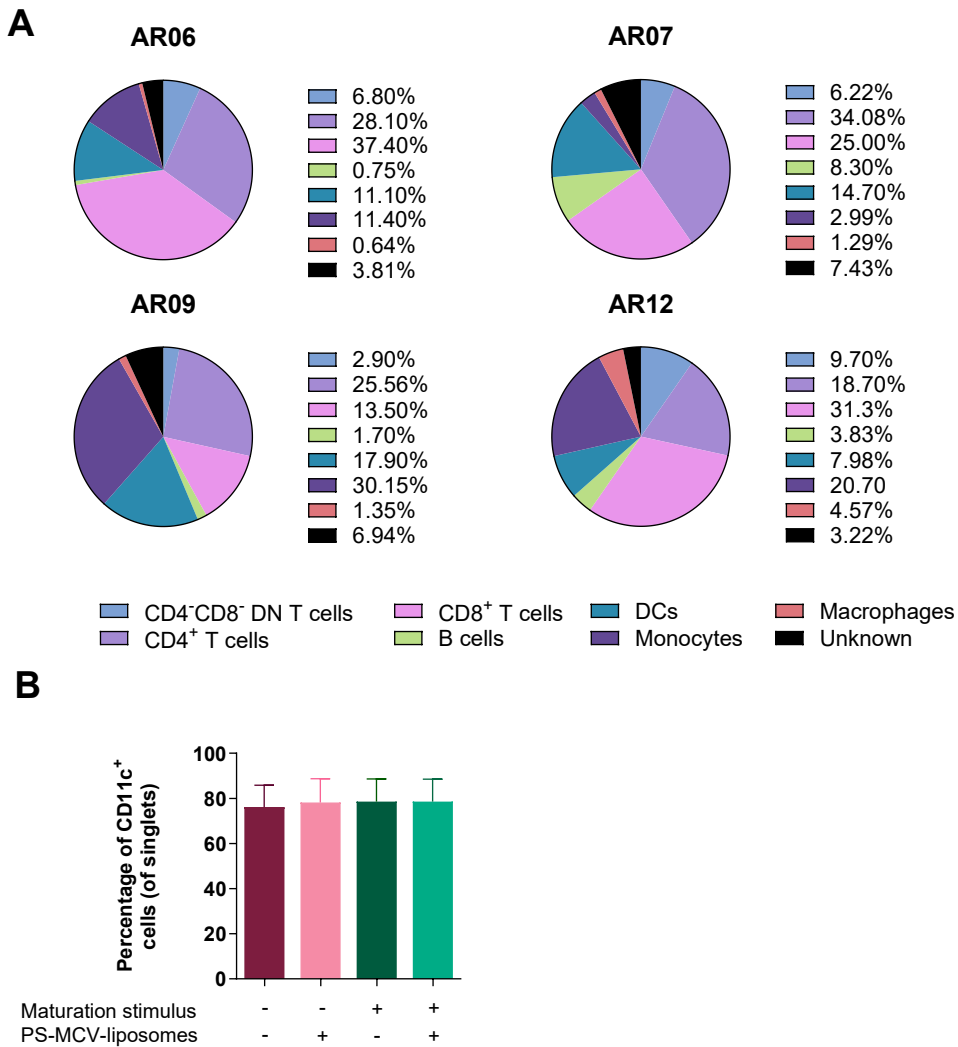
HLA-DR					HLA-DQ				
D	Nº of proteins	Nº of peptides	Nº of AChR peptides		D	Nº of proteins	Nº of peptides	Nº of AChR peptides	
			High/medium	Low				High/medium	Low
1	150	623	ND	ND	1	41	74	ND	ND
2	231	911	ND	1	2	51	101	ND	ND
4	210	749	2	ND	4	5	6	ND	ND
6	320	1121	3	ND	5	93	380	1	ND
7	228	726	ND	1	6	51	79	ND	ND
8	309	1112	ND	ND	7	37	62	ND	ND
10	250	968	ND	ND	8	91	226	ND	ND

D: Donor; ND: not detected

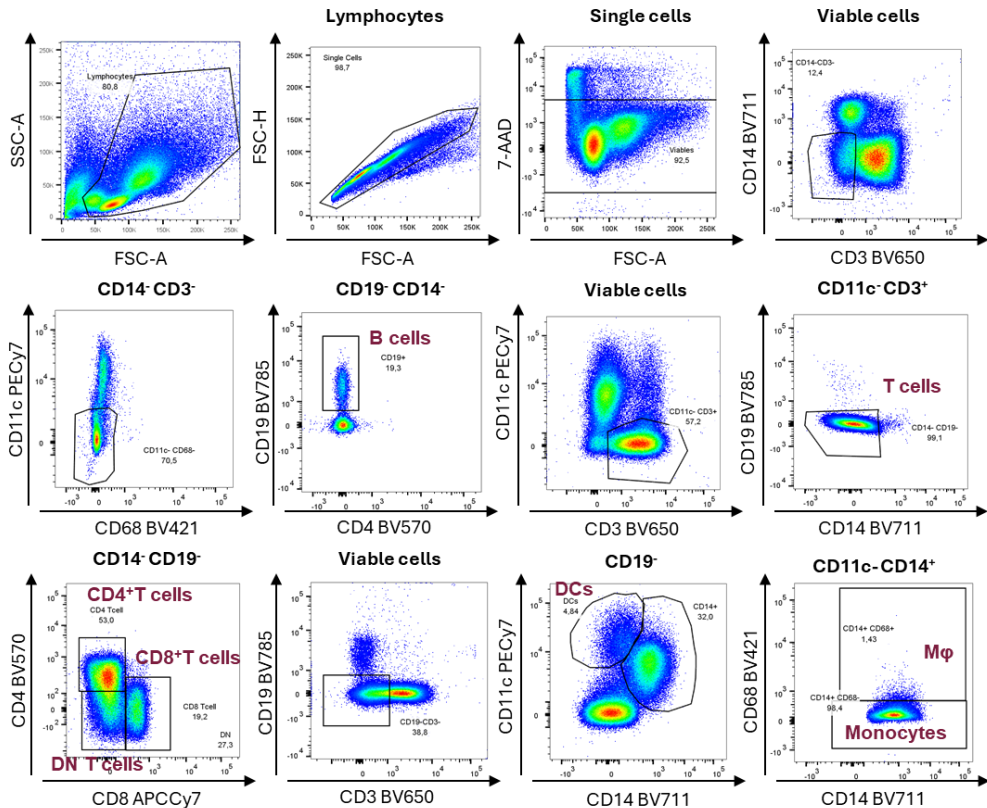
Supplementary Table 6. Summary of the number peptides presented by HLA class II molecules identified in SPACER peptide conditions.

HLA-DR					HLA-DQ				
D	Nº of proteins	Nº of peptides	Nº of SPACER peptides		D	Nº of proteins	Nº of peptides	Nº of SPACER peptides	
			High/medium	Low				High/medium	Low
6	184	615	60	ND	10	79	347	81	ND
8	269	847	14	ND					
9	340	1643	115	ND					
10	268	1185	70	ND					

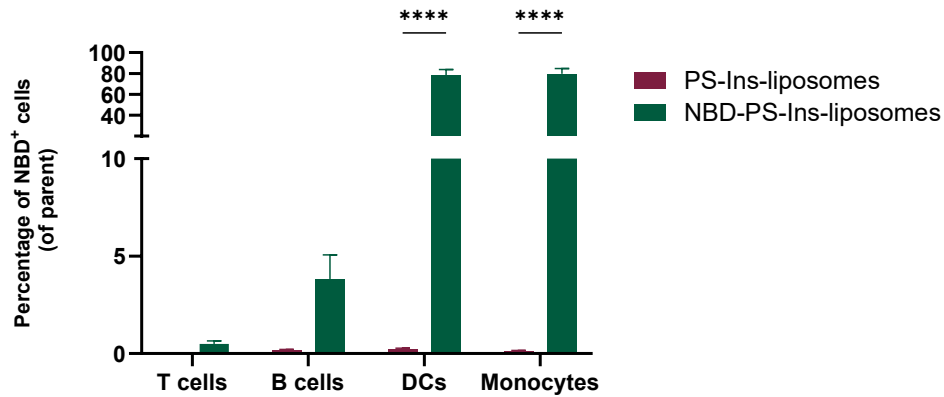
D: donor; ND: not detected



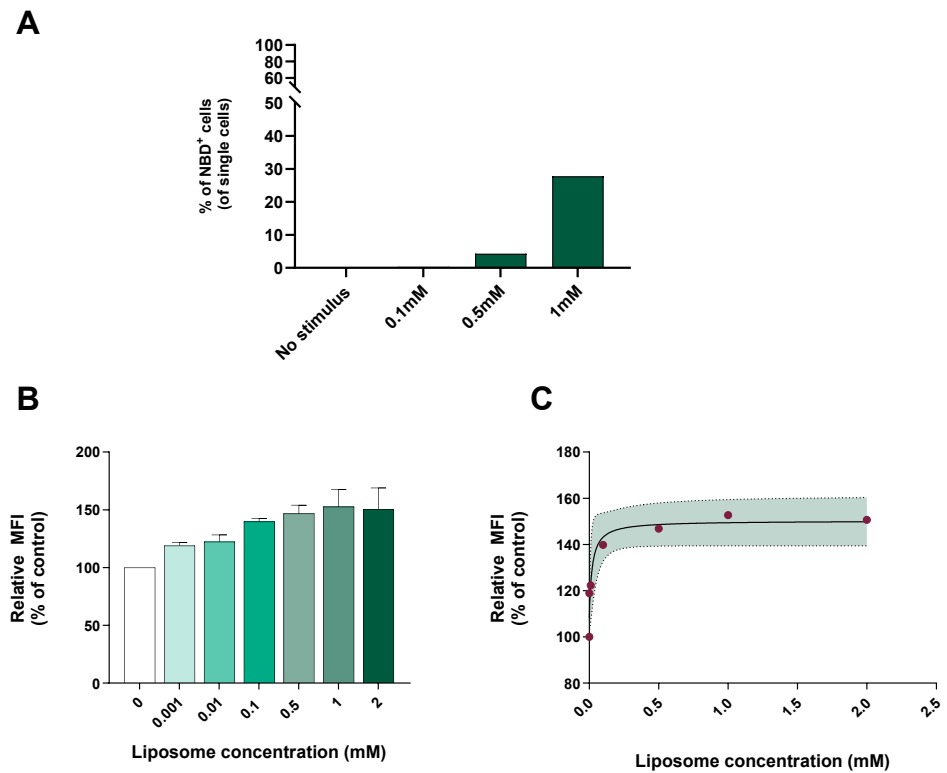
Supplementary Figure 25. Synovial fluid immune cell subset data from RA patients. (A) Synovial fluid mononuclear immune cell subset composition of four RA patients the day of the extraction of the sample. **(B)** Percentage of CD11c⁺ cells after culture of CD14⁺ isolated fraction of SFMCs with GM-CSF and IL-4 for 6 days (n=3). Bars represent iDCs (dark pink), iDCs after PS-loaded-liposome phagocytosis (light pink), mDCs (dark green) or mDCs after PS-loaded-liposome phagocytosis (light green). Data represented as mean ± SEM.



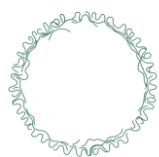
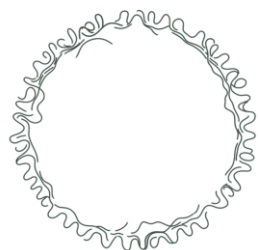
Supplementary Figure 26. Flow cytometry strategy for analysing PBMCs and SFMCs from patients' and healthy donors' biological samples. Representative gating strategy used to analyse immune cell subsets within SFMCs. Cells were first gated for lymphocytes (FSC-A vs SSC-A), singlets (FSC-A vs FSC-H) and viable cells (7-AAD⁻ cells). Double negative CD14 and CD3 cells were gated to discard the CD11c⁻ subset and finally select CD19⁺ cells, defined as B cells. From viable cells, double CD19 and CD14 negative cells were gated to obtain CD3⁺ cell, defined as T cells, which were gated select CD4⁺ or CD8⁺ T cells. From viable cells, double negative CD19 and CD3 cells were gated to obtain CD11c⁺ but CD14⁻ cells, defined as DCs. CD14⁺ subset was gated to select CD68⁺ cells, defined as Mφ, and CD68⁻ cells, defined as monocytes. FMOs of CD68, and CD4 were used to define the gates (not shown).



Supplementary Figure 27. Percentage of NBD positive cells after 24h incubation of NBD-labelled PS-Ins-liposomes with PBMCs from healthy controls (n=3). Bars represent conditions incubated with non-fluorescent PS-Ins-liposomes (dark pink) or conditions incubated with NBD-PS-Ins-liposomes (dark green). Data represented as mean $\pm$ SEM. Significant differences were found when comparing among NBD positive or negative cells from the same cell subset (**** p <0.0001, Two-way ANOVA).



Supplementary Figure 28. TANOUE cells interacting with PS-AChR-liposomes present a dose-response modulation in TOLX expression levels. (A) NBD positivity of TANOUE cells after being incubated with different concentrations of NBD-labelled PS-loaded-liposomes. **(B)** Relative MFI of TOLX in B cells after interaction with a gradient of PS-AChR-liposome concentrations (n=3) **(C)** and the correspondent regression curve performed with the median of each condition. Bars represent with a gradual scale from white to dark green the increasing concentration of PS-AChR-liposomes. Data is presented as a mean $\pm$ SD.





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