Universitat Autònoma de Barcelona

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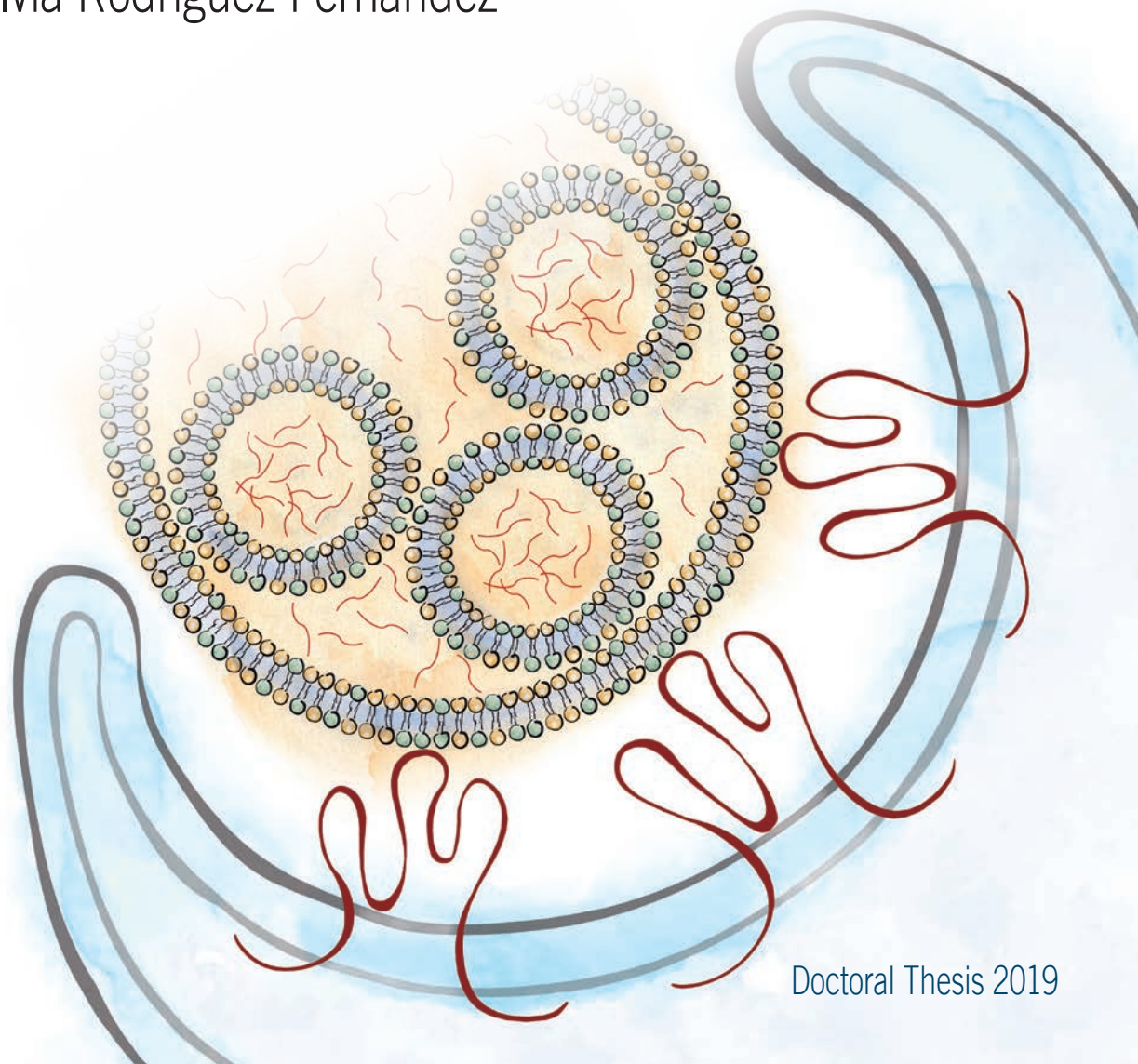

Doctoral Thesis 2019

Phosphatidylserine-rich liposomes to tackle autoimmunity

En route to translationality

Silvia Rodríguez Fernández

DOCTORAL THESIS
Badalona, 28th June 2019

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PhD programme in Advanced Immunology
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Universitat Autònoma de Barcelona

Phosphatidylserine-rich liposomes to tackle autoimmunity

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Liposomes rics en fosfatidilserina per tractar l'autoimmunitat
En camí cap a la translacionalitat

Liposomas ricos en fosfatidilserina para tratar la autoinmunidad
En camino hacia la translacionalidad

Thesis presented by Silvia Rodríguez Fernández to qualify for the
PhD degree in Advanced Immunology by the Universitat Autònoma de Barcelona.

The presented work has been performed in the Immunology of Diabetes group,
at the Germans Trias i Pujol Health Sciences Research Institute (IGTP),
and directed by Dr Marta Vives Pi.

Badalona, 28th June 2019

Silvia Rodríguez Fernández
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La Dra. Marta Vives Pi, investigadora biomèdica de la Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol i professora associada del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona,

Certifica:

Que el treball experimental i la redacció de la memòria de la Tesi Doctoral ***'Phosphatidylserine-rich liposomes to tackle autoimmunity. En route to translationality'*** han estat realitzats per na Silvia Rodríguez Fernández sota la seva direcció i considera que és apta per ser presentada per optar al grau de Doctor en Immunologia Avançada per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signa aquest document a Badalona,
el 20 de maig del 2019.

Dra. Marta Vives Pi

A mis padres,
Vicente y Carmen.
Y a Ferran

"Education never ends, Watson.
It is a series of lessons with the greatest for the last."

His Last Bow
Sir Arthur Conan Doyle

TABLE OF CONTENTS

ABBREVIATIONS

7-AAD	7-amino-actinomycin D
ACHT	Adrenocorticotropic hormone
AF	Alexa fluor
AIRE	Autoimmune regulator
ALT	Alanine aminotransferase
APC	Allophycocyanin
APCCy7	Allophycocyanin-cyanine 7
APCs	Antigen-presenting cells
AUC	Area under the curve
BAI1	Brain-specific angiogenesis inhibitor 1
BB	BioBreeding
BBB	Blood-brain barrier
BCR	B cell receptor
BMI	Body mass index
CBA	Cytometric bead array
CC	Cytokine cocktail
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CH	Cholesterol
CIS	Clinically isolated syndrome
CNS	Central nervous system
Cryo-TEM	Cryogenic transmission electron microscopy
CSF	Cerebrospinal fluid
CTLA	Cytotoxic T lymphocyte antigen
CTV	Cell-trace violet
DCs	Dendritic cells
DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
EAE	Experimental autoimmune/allergic encephalomyelitis
EDSS	Expanded disability status scale

eF660	eFluor 660
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FC	Fold change
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FoxP3	Forkhead box P3
FSC	Forward scatter
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
GLUT-2	Glucose transporter 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glucose-6-phosphatase isomerase-derived peptide
GWAS	Genome-wide association studies
HbA1c	Glycated haemoglobin or haemoglobin A1c
HDL	High-density lipoprotein
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
HSP	Heat shock protein
i.d.	Intradermal
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
IA-2	Insulinoma-antigen 2
iDCs	Immature dendritic cells
Idd	Insulin-dependent diabetes
IDDAA1c	Insulin dose-adjusted haemoglobin A1c
IDDM	Insulin-dependent diabetes mellitus
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGRP	Glucose-6-phosphatase catalytic subunit-related protein
IL	Interleukin
iNKT	Invariant natural killer cell
IU	International units
LPS	Lipopolysaccharide
MBP	Myelin basic protein
mDCs	Mature dendritic cells
MDLN	Mediastinal lymph nodes
MFI	Median of fluorescence intensity

MHC	Major histocompatibility complex
miRNA	MicroRNA
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
myDCs	Myeloid dendritic cells
NEDA	No evidence of disease activity
NK	Natural killer cells
NOD	Non-obese diabetic
nPOD	Network for pancreatic organ donors with diabetes
OG488	Oregon green 488
PB	Pacific blue
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PC-liposomes	Phosphatidylserine-free liposomes
PCNRP-liposomes	PC-liposomes encapsulating NRP peptide
PCOG488-liposomes	Empty oregon green 488-fluorescent PC-liposomes
PD	Programmed death
pDCs	Plasmacytoid dendritic cells
PdI	Polydispersity index
PE	Phycoerythrin
PECy5	Phycoerythrin-cyanine 5
PECy7	Phycoerythrin-cyanine 7
PGE_2	Prostaglandin E_2
PLN	Pancreatic lymph nodes
PLP	Proteolipid protein
pMHC	Peptide-major histocompatibility complex
PML	Progressive multifocal leukoencephalopathy
PPAR	Peroxisome proliferator-activated receptor
PPMS	Primary progressive multiple sclerosis
PR	Partial remission
PRMS	Progressive-relapsing multiple sclerosis
PS	Phosphatidylserine
PS2.5mi-liposomes	PS-liposomes encapsulating 2.5mi peptide
PS30-liposomes	PS-liposomes at 30 mM of lipid concentration
PS90-liposomes	PS-liposomes at 90 mM of lipid concentration
PSAB-liposomes	Mix of 50% PSA-liposomes and 50% PSB-liposomes
PSA-liposomes	PS-liposomes encapsulating human insulin A chain
PSB-liposomes	PS-liposomes encapsulating human insulin B chain

PS-liposomes	Phosphatidylserine-rich liposomes
PSMOG-liposomes	PS-liposomes encapsulating MOG peptide
PSNRP-liposomes	PS-liposomes encapsulating NRP peptide
PSOG488-liposomes	Empty oregon green 488-fluorescent PS-liposomes
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end-products
RINe	RNA integrity number
RNA-seq	RNA-sequencing
RPKM	Reads per kilobase of transcript per million reads mapped
RR	Relapsing-remitting
RRMS	Relapsing-remitting multiple sclerosis
s.c.	Subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
SPF	Specific-pathogen free
SPMS	Secondary-progressive multiple sclerosis
SSC	Side scatter
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCR	T cell receptor
TEC	Thymic epithelial cells
TEDDY	The environmental determinants of diabetes in the young
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TIM	T cell immunoglobulin mucin domain
TLRs	Toll-like receptor
TMEV	Theiler’s murine encephalomyelitis virus
TNF	Tumour necrosis factor
TolDCs	Tolerogenic dendritic cells
TR1	Type 1 regulatory T cells
Treg	Regulatory T lymphocytes
V450	Violet 450
VNTR	Variable number tandem repeat
WHO	World health organisation
ZnT8	Zinc transporter 8

SUMMARY

Autoimmune diseases are caused by defective immunological tolerance, and reportedly affect up to 10% of the global population. In the last years, current medical interventions have transformed these disorders into chronic and manageable, but they still entail high rates of morbidity and mortality. Hence, there is an urgent need to develop therapies capable of restoring the breach of tolerance selectively, which halt the autoimmune aggression and allow the regeneration of the targeted tissue. In physiological conditions, the phagocytosis of apoptotic cells performed by phagocytes such as dendritic cells (DCs) —a process termed efferocytosis— prompts the acquisition of tolerogenic features and the ability to restore tolerance. Indeed, a cell immunotherapy consisting of DCs rendered tolerogenic (tolDCs) by apoptotic β-cell efferocytosis arrested the autoimmune attack against β-cells in an experimental model of type 1 diabetes (T1D). However, in light of the hurdles in obtaining and standardising human autologous apoptotic β-cells for its implementation in the clinics, a nanotherapeutic strategy based on liposomes mimicking apoptotic cells was designed. The fundamental characteristics of these synthetic vesicles are: a high percentage of phosphatidylserine (PS) —phospholipid unique to the apoptotic cell membrane—, diameter superior to 500 nm, negative charge and efficient encapsulation of insulin peptides. Importantly, this strategy was equally effective in inducing tolDCs and blunting β-cell autoimmunity as the immunotherapy based on apoptotic cells.

The hypothesis of this work is that autoantigen-loaded PS-liposomes can re-establish tolerance in several antigen-specific autoimmune diseases through the induction of tolDCs and the expansion of regulatory T lymphocytes, and that they have translational potential to tackle human autoimmune disorders. The main aim of the present work has been to characterise the tolerogenic potential of PS-liposomes globally. To this end, different autoantigenic peptides relevant in autoimmune diseases have been efficiently encapsulated into PS-liposomes, without difficulties in preserving their appropriate diameter and charge, thus demonstrating the versatility of the therapy to different autoimmune pathologies. In the experimental model of T1D, the administration of PS-liposomes causes the expansion of clonal $CD4^+$ regulatory T cells and $CD8^+$ T cells, which contribute to the long-term re-establishment

of tolerance. Moreover, in the same model, the biocompatibility and safety of the final product have been confirmed given its optimal tolerability. Furthermore, PS-liposomes have been adapted to the experimental multiple sclerosis model by merely replacing the encapsulated autoantigen. In this model, PS-liposomes elicit the generation of tolDCs and decrease the incidence and severity of the disease correlating with an increase in the frequency of regulatory T cells, a fact that validates the potential of PS-liposomes to serve as a platform for tolerance re-establishment in different autoimmune diseases. Finally, considering its future clinical implementation, the effect of the PS-liposomes therapy has been determined in human DCs obtained from patients with T1D. In DCs from adult patients, PS-liposomes are efficiently phagocyted by DCs with rapid kinetics dependent on the presence of PS, and this induces a tolerogenic transcriptome, phenotype and functionality that are similar to those observed in experimental models. However, DCs from paediatric patients display defects in their phagocytic capacity correlating with the time of disease progression, albeit their phenotype and immunoregulatory gene expression after PS-liposomes phagocytosis point to an optimal tolerogenic ability.

In conclusion, the liposomal immunotherapy herein described, which is based on efferocytosis as a powerful tolerance-inducing mechanism, achieves apoptotic mimicry in a simple, safe and efficient manner. Additionally, liposomes offer advantages in terms of production and standardisation. Therefore, PS-liposomes possess translational potential and constitute an encouraging strategy to restore immunological tolerance in antigen-specific autoimmune diseases.

RESUM

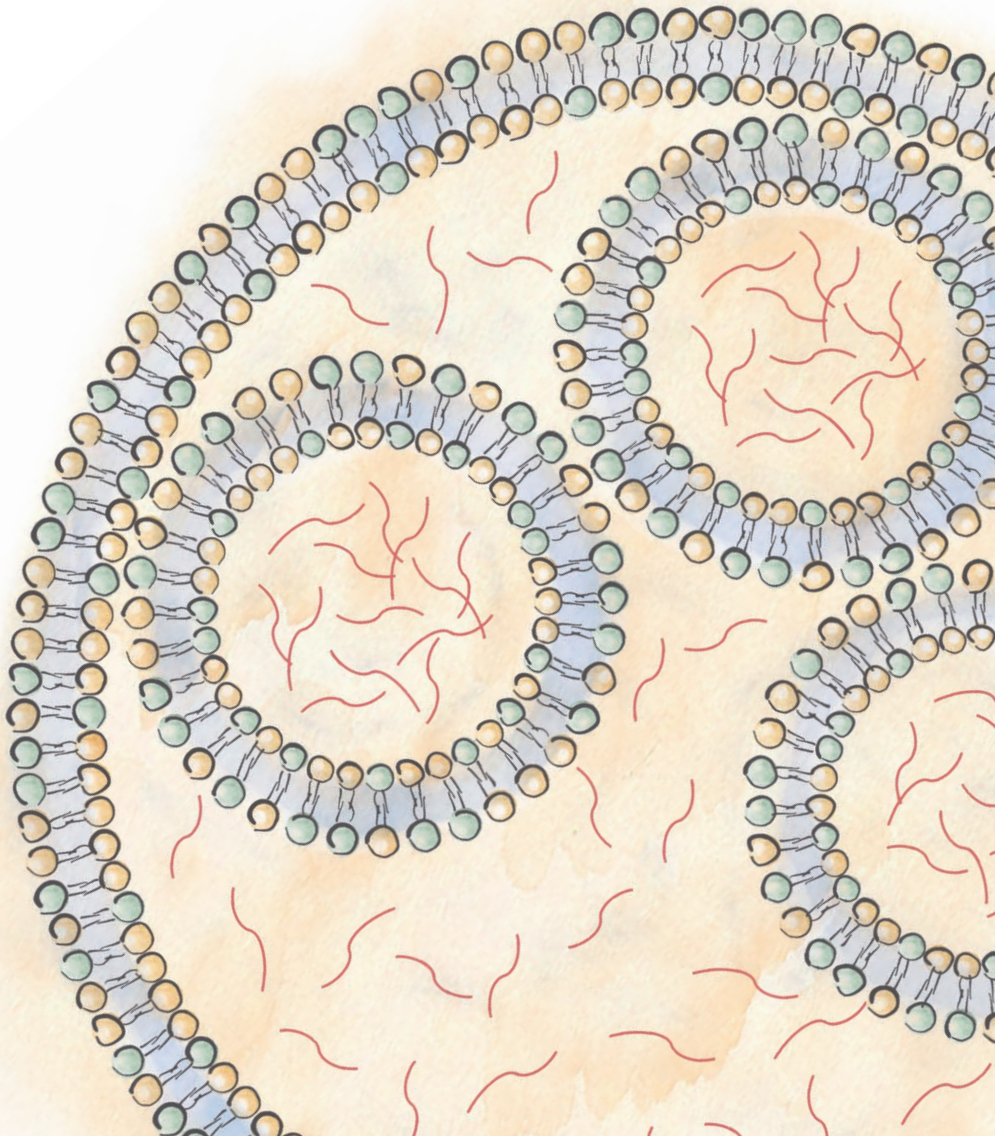
Les malalties autoimmunitàries estan causades per defectes en la tolerància immunològica, i afecten a gairebé un 10% de la població. Darrerament, diverses intervencions mèdiques han convertit aquestes malalties en cròniques, però el seu diagnòstic encara comporta morbiditat i mortalitat elevades. Així, un repte biomèdic urgent és el desenvolupament de teràpies que puguin restablir selectivament la tolerància, aturin l'atac autoimmunitari i permetin la regeneració del teixit danyat. En condicions fisiològiques, la fagocitosi de cèl·lules apoptòtiques per part de fagòcits com les cèl·lules dendrítiques (CDs) —procés que rep el nom d'eferocitosi— els indueix propietats tolerogèniques i l'habilitat de restaurar la tolerància. Una demostració d'això és que una immunoteràpia cel·lular consistent en CDs tolerogèniques (CDtols) degut a l'eferocitosi de cèl·lules β apoptòtiques va aturar l'atac autoimmunitari contra les cèl·lules β en un model experimental de diabetis tipus 1 (DT1). Donades les dificultats en obtenir i estandarditzar cèl·lules β apoptòtiques autòlogues humanes per la seva implementació clínica, es va dissenyar una nanoteràpia basada en liposomes que simulen cèl·lules apoptòtiques. Les principals característiques d'aquestes vesícules sintètiques són: elevat percentatge de fosfatidilserina (FS) —fosfolípid característic de la membrana de les cèl·lules apoptòtiques—, diàmetre major de 500 nm, càrrega negativa i eficient encapsulació de pèptids d'insulina. Aquesta estratègia és tant efectiva en generar CDtols i aturar l'autoimmunitat contra les cèl·lules β com la immunoteràpia basada en cèl·lules apoptòtiques.

La hipòtesi d'aquest treball és que els FS-liposomes poden restablir la tolerància en diverses malalties autoimmunitàries antigen-específiques mitjançant la generació de CDtols i l'expansió de limfòcits T reguladors, i que tenen el potencial translacional per abordar patologies autoimmunitàries humanes. L'objectiu principal d'aquest estudi ha estat caracteritzar globalment el potencial tolerogènic dels FS-liposomes. Amb aquesta finalitat, diferents pèptids autoantigènics rellevants en malalties autoimmunitàries s'han encapsulat en FS-liposomes de manera eficient i sense dificultats en mantenir el diàmetre i la càrrega, demostrant la versatilitat de l'estratègia a diferents patologies autoimmunitàries. En el model experimental de DT1, l'administració de FS-liposomes ha expandit clons de cèl·lules T $CD4^+$ reguladores i T $CD8^+$

que contribueixen a l'efecte tolerogènic de la teràpia a llarg termini. En el mateix model, s'ha demostrat la biocompatibilitat i seguretat del producte final donada la seva òptima tolerabilitat. D'altra banda, els FS-liposomes s'han adaptat al model d'esclerosi múltiple experimental simplement reemplaçant el pèptid encapsulat. En aquest model, els FS-liposomes han induït CDtols i han reduït la incidència i severitat de la malaltia correlacionant amb un increment en la freqüència de cèl·lules T reguladores, fet que valida el potencial dels FS-liposomes a constituir una plataforma per a la recuperació de tolerància en diferents malalties autoimmunitàries. Finalment, i de cara a una futura implementació clínica, s'han determinat els efectes de la teràpia en CDs humanes obtingudes de pacients amb DT1. En CDs de pacients adults, els FS-liposomes han estat fagocitats eficientment per les CDs amb una ràpida cinètica dependent de la FS, i això ha causat l'adquisició d'un transcriptoma, fenotip i funcionalitat tolerogènics similars als observats en els models experimentals. En CDs de pacients pediàtrics, però, les CDs han presentat defectes en la seva capacitat fagocítica correlacionant amb el temps de progressió de la malaltia; tanmateix, el seu fenotip i expressió de gens immunoreguladors després de la fagocitosi dels FS-liposomes són indicatius d'una habilitat tolerogènica òptima.

En conclusió, la immunoteràpia liposomal descrita, que es basa en l'eferocitosi com a mecanisme inductor de tolerància, assoleix el mimetisme apoptòtic de manera simple, segura i eficient. A més, els liposomes ofereixen avantatges quant a producció i estandardització. Per tant, els FS-liposomes tenen potencial translacional i constitueixen una estratègia prometedora per recuperar la tolerància immunològica en malalties autoimmunitàries antigen-específiques.

INTRODUCTION



1. The fine line between self-tolerance and self-reactivity

Cells, tissues and organs of the immune system work coordinated to protect the host against a vast range of pathogens and potentially life-threatening cellular processes. When engaged, the immune response must be quick to rise, specifically directed to hazardous components, diverse to cover all manners of specificities, and capable of restraining itself when it is no longer necessary. Also, the immune system possesses memory, to fight more intensely and rapidly against those threats that have entered the body before. Importantly, the immune system is also able to establish and maintain a lack of reactivity against the antigens of the host (self-antigens), a phenomenon that is termed *immunological tolerance* (Waldmann, 2014). Tolerance to self-antigens, named *self-tolerance*, is an essential property of the immune system and is preserved by several tightly-regulated processes, which include the elimination of lymphocytes that possess self-antigen-specific receptors, the inactivation of self-reactive lymphocytes, and the suppression of these cells by the actions of other regulatory cells or regulatory mediators. The failure of these mechanisms can give rise to pathogenic immune reactions against self-antigens —called *self-reactivity* or *autoimmunity*—, which, if severe enough to cause disease, could ultimately lead to the development of an autoimmune disorder.

1.1. Key regulators

Immunological tolerance was first described by Owen and colleagues in 1945, when they demonstrated that dizygotic cattle twins possessed significant numbers of red blood cells from the other twin in their circulation (Owen, 1945). Surprisingly, the immune system of the cattle did not react against these foreign antigens, whereas non-twin adult cows did mount an antibody response when injected with red blood cells from another cow. Four years later, Burnet and Fenner cited Owen's studies as evidence of the phenomenon they had termed *tolerance* (Burnet and Fenner, 1949). In their book, they proposed that antigens present during the perinatal period were

acknowledged as “self”, and that they established an unresponsive state that thwarted the development of cognate immune responses when the host reached immunological maturity. By contrast, antigens encountered after that period would induce an immune response. Hence, the immune response was acknowledged as being able to differentiate between “self” and “non-self”. In the early 1950s, Billingham and Medawar further confirmed that tolerance was *adoptively acquired* after a first interaction with the antigen during the foetal period, by showing that injections of allogeneic lymphoid cells into neonatal mice were immunologically tolerated (Billingham *et al.*, 1953, 1954). Moreover, these observations served as foundation for Burnet’s *clonal selection hypothesi*s (Burnet, 1957), which proposed that antigen-specific receptors were present on the surface of lymphocytes and induced an effector response when a foreign antigen was bound, contrary to the clonal deletion elicited when occupied by any antigen learned as *self* during development. These four decisive reports, whose importance could not be overstated, paved the way to our current understanding of immunological tolerance, and merited the Nobel Prize in Physiology or Medicine awarded to Burnet and Medawar in 1960.

In the preservation of self-tolerance, cells from both the innate and the adaptive immune system are essential. In fact, tolerance is driven in an antigen-specific manner, and the conditions of the antigen exposure and presence or absence of concomitant stimuli determine whether an antigen induces an immunogenic or a tolerogenic response. In exceedingly broad terms, it could be stated that antigen presenting cells (APCs), namely dendritic cells (DCs), macrophages and B cells, are responsible for the induction of tolerance, whereas T lymphocytes are the ones to receive this education and enforce it. Logically, this is a comprehensive definition and does not fully grasp the complex reality that is embedded in immunological tolerance. Indeed, multiple checkpoints control that autoimmunity has slim chances to occur, and they are broadly named as central tolerance and peripheral tolerance mechanisms.

1.1.1. Central tolerance

Central tolerance is executed in central lymphoid organs, with the thymus being responsible for central T cell tolerance and the bone marrow carrying out central B cell tolerance. Both processes are necessary to eliminate lymphocytes with high-affinity receptors for self-antigens, which are generated randomly by the rearrangement of the T cell receptor (TCR) gene segments on T cells, and the B cell receptor (BCR) gene segments on B cells. This process guarantees that the adaptive division of the immune response will be as diverse as possible to recognise and neutralise all manners of hazards, but it also provides better opportunities for autoreactive lympho-

cytes to be generated. Thus, central tolerance ensures that the *repertoire* of mature naïve lymphocytes cannot react against self-antigens.

In the thymus, the maturation of T cells involves a two-step process to, firstly, positively select those thymocytes having a TCR capable of binding to Major Histocompatibility Complex (MHC) molecules with low affinity and, secondly, to delete those thymocytes bearing a TCR with high affinity for self-MHC/peptide complexes (Xing and Hogquist, 2012). It is noteworthy that only 2 to 5% of thymocytes complete their maturation and exit the thymus, whereas the vast majority die by apoptosis within. Thus, immature double positive $CD4^+CD8^+$ T lymphocytes enter the thymic cortex, where they browse the surface of cortical thymic epithelial cells (TECs) until they can bind an MHC/peptide complex, thereby receiving survival signals through their TCR. If thymocytes are unable to find a complex to bind, either because their TCR is not functional or does not have sufficient affinity, they die by neglect. This positive selection results in an MHC restriction, which triggers their commitment into single positive $CD4^+$ or $CD8^+$ T cells and their migration to the thymic medulla. There, thymocytes undergo a negative selection process, which consists of clonal deletion once they bind to an MHC/peptide complex with high affinity and receive costimulatory signals. APCs residing in the thymus, such as DCs, macrophages and B cells, can mediate negative selection, but medullary TECs possess a unique feature to aid in this process (Klein *et al.*, 2014). These cells are able to express the Autoimmune Regulator (AIRE) protein, which is a transcriptional regulator that controls the ectopic expression of tissue-restricted antigens that would not be expressed in the thymus otherwise (Chan and Anderson, 2015). Therefore, thymocytes recognising these self-antigens with high affinity are deleted, whereas those with low or intermediate affinity end their maturation successfully. Interestingly, some self-reactive $CD4^+$ T cells with intermediate or high affinity recognition for self-antigens can differentiate into regulatory T (Treg) cells, which are called thymic or natural Treg cells and express the transcription factor forkhead box P3 (FoxP3) (Lee and Lee, 2018). When their maturation is complete, they leave the thymus to patrol the body and act in peripheral tolerance mechanisms. To date, what drives the choice between deletion and differentiation into Treg cells is not fully understood, although the presence of interleukin (IL)-2 and IL-15 could contribute to this commitment (Apert *et al.*, 2017).

In the bone marrow, immature B cells bearing a self-reactive BCR can be deleted through BCR-mediated induction of apoptosis or edit their BCR through the reactivation of *RAG* genes (Nemazee, 2017). These two mechanisms take place when B cells receive strong signals through their BCR, which happens when receptors cross-link by binding to self-antigens on the surface of stromal cells. However, when B cells recognise soluble self-antigens, bind antigens with low affinity or receptors do

not cross-link, the signal received by the B cell is weak. This type of signal leads to the downregulation of the BCR expression and a block in antigen-receptor signalling, which is otherwise known as anergy. Then, the B cells exit the bone marrow in this unresponsive state.

As with any other process, central tolerance is not always reliable, and a significant number of autoreactive cells can complete their maturation and egress from these organs into the circulation. Therefore, mechanisms of peripheral tolerance are absolutely necessary to block the activation of these potentially harmful cells.

1.1.2. Peripheral tolerance

The mechanisms that mediate peripheral tolerance occur mainly in secondary lymphoid organs, such as lymph nodes or the spleen, or at the tissue where the relevant antigen is expressed. The processes involved in peripheral T cell tolerance include (Mueller, 2010):

- **Anergy.** The state of unresponsiveness in T cells can be induced by recognition of the antigen in the absence of costimulatory signals. This state is maintained in T cells by the blocking of TCR-induced signalling, either by the degradation of the TCR-associated proteins or the recruitment of inhibitory molecules to the TCR complex, such as the cytotoxic T lymphocyte antigen (CTLA)-4 or the programmed death (PD)-1.
- **Deletion.** T lymphocytes that recognise self-antigens with high affinity, do so in the absence of costimulatory signals, or undergo repeated stimulation, can die by apoptosis.
- **Treg cell-mediated action.** Naïve T cells, in the periphery, can be induced to express FoxP3 and become Treg cells, and this can take place when T cells recognise antigen without any additional stimuli (e.g. costimulation) or when they are exposed to the antigen in the context of inhibitory cytokines such as IL-10 or transforming growth factor (TGF)-β. Similarly, antigen-stimulation can induce the generation of type 1 regulatory (TR1) cells, which are $FoxP3^{-}CD4^{+}$ Treg cells with the ability to regulate effector responses in an antigen-specific manner (White and Wraith, 2016). Along with thymic Treg cells, they are responsible for the maintenance of self-tolerance through the following mechanisms:
  - *Effector T cell suppression.* To do so, Treg cells produce anti-inflammatory IL-10 and TGF-β cytokines, reduce the ability of APCs to stimulate T cells by linking their CTLA-4 molecule to the costimulatory molecules on APCs, deprive the media of IL-2 due to their high expression of IL-2 receptor α chain (CD25), and may kill effector T cells by secreting perforin and granzyme (Arce-Sillas *et al.*, 2016).

– *Infectious tolerance.* Treg cells can confer their modulating abilities to naïve $CD4^+$ T lymphocytes by either cell-to-cell contact accompanied by TGF-β secretion or by altering the ability of APCs to mature and costimulate immune response (Gravano and Vignali, 2012).
– *Bystander suppression.* Treg cells can suppress the response of other antigen-specific $CD4^+$ and $CD8^+$ T lymphocyte clones by cell-to-cell contact (Sakaguchi *et al.*, 2009; Thornton and Shevach, 2000).

As for B cells, once leaving the bone marrow, mature B cells that recognise self-antigens in the absence of T cells become anergic or die by apoptosis. Also, B cells are prevented from responding to self-antigens that only engage the BCR by inhibitory receptors that set a threshold for B cell activation (Yasuda *et al.*, 2017). As is the case of Treg cells, regulatory B cells also contribute to the maintenance of self-tolerance through the production of high amounts of IL-10 and TGF-β and the expression of inhibitory molecules to suppress T cell and B cell responses in a contact-dependent manner (Yang *et al.*, 2013).

1.1.3. Efferocytosis as a tolerance-inducing mechanism

Another efficient mechanism in inducing tolerance is apoptotic cell clearance, a process that is termed efferocytosis. In the human body, more than 100 billion cells undergo programmed cell death or apoptosis every day, stemming from processes of cell turnover, cell damage, infection or immunological tolerance (Arandjelovic and Ravichandran, 2015). In the signalling pathway of apoptosis, mitochondrial factors are released into the cytoplasm, the cell and nuclei condense to become fragmented and the plasma membrane forms blebs. The most critical factor of apoptosis is that neither the nuclear envelope or the plasma membrane lose their functional integrity (Elliott and Ravichandran, 2010), thus preventing the leakage of intracellular damage-associated molecules or immunogenic contents to the extracellular space. This fact allows death by apoptosis to be devoid of inflammation, and this is essential in the maintenance of homeostasis. Moreover, apoptotic cells are rarely visible in physiological conditions due to the high rate of apoptotic cell clearance carried out by phagocytes.

Efferocytosis is performed in four steps (Fond and Ravichandran, 2016). Firstly, apoptotic cells rapidly attract phagocytes to their corpses through *Find me* signals, which include the exposure of specific molecules on their surface, such as adhesion molecules like ICAM-3 (Torr *et al.*, 2012), and the release of soluble molecules like CX_3CL_1 and nucleotides (Elliott *et al.*, 2009; Truman *et al.*, 2008). Also, *Stay away* signals are released to block the recruitment of other inflammatory cells,

such as neutrophils, in order to maintain a proper anti-inflammatory environment (Bournazou *et al.*, 2009). Afterwards, the exposure of *Eat me* signals on the surface of apoptotic cells prompts the engulfing action of phagocytes (Hochreiter-Hufford and Ravichandran, 2013). The most renowned *Eat me* signal is phosphatidylserine (PS), a phospholipid that is usually kept in the inner leaflet of the lipid bilayer in viable cells and translocated to the outer leaflet when apoptosis takes place. In the phagocyte, recognition of PS is accounted by a high number of receptors that can bind PS, either directly or indirectly. The former include molecules like T cell immunoglobulin mucin domain (TIM)-1, TIM-3, TIM-4, the CD300 protein family, Stabilin-1 and Stabilin-2, brain-specific angiogenesis inhibitor 1 (BAI1), receptor for advanced glycation end-products (RAGE) and scavenger receptors like CD36 and CD68, among others, whereas the latter are comprised of complexes formed by TAM receptors (Tyro-3, Axl, and MerTK) and Gas6/Protein S or MFG-E8 and αvβ3/5 integrin (**Figure 1A**, Moller-Tank and Maury, 2014; Vives-Pi *et al.*, 2015). Moreover, apoptotic cells downregulate the expression of CD47 —a *Don't eat me* signal usually present on the surface of normal viable cells— to allow phagocytosis. Finally, within the phagocyte, signalling pathways regulate the cytoskeletal rearrangement for the engulfment of the apoptotic cell, its digestion and the processing of autoantigens to induce tolerance (Hochreiter-Hufford and Ravichandran, 2013). Indeed, after efferocytosis, phagocytes —essentially, DCs— are compelled into a tolerance-inducing state in which their immunogenicity is suppressed. This means that they are unable to mature and upregulate the expression of antigen-presenting molecules (MHC class II) and costimulatory molecules (CD40, CD80, CD83, CD86). Also, they produce high amounts of immune-modulatory mediators such as IL-10, TGF-β, prostaglandin E_2 (PGE_2) and indoleamine 2,3-dioxygenase (IDO) and low amounts of pro-inflammatory cytokines like IL-1, IL-6, IL-12 and tumour necrosis factor (TNF)-α, and have decreased ability to stimulate T cell proliferation and expand Treg cells (Domogalla *et al.*, 2017; Szondy *et al.*, 2017). Paradigmatically, all these features aim to promote immune tolerance to autoantigens (**Figure 1B**, Erwig and Henson, 2007; Hopp *et al.*, 2014). Hence, DCs displaying this "tolerogenic" behaviour are so-called tolerogenic DCs (tolDCs).

On the other hand, if apoptotic cells are not engulfed within a definite short time —either by a heightened apoptosis rate or by little removal capacity of phagocytes—, they become late apoptotic cells —termed secondary necrotic cells. This process entails the loss of their membrane integrity (Silva, 2010) and favours the release of intracellular content and danger signals, promoting the generation of a pro-inflammatory environment (Kimani *et al.*, 2014). This setting prompts the maturation of DCs and allows them to present autoantigens along with costimulatory signals, thus triggering immunological recognition and, potentially, autoimmunity (**Figure 1C**).

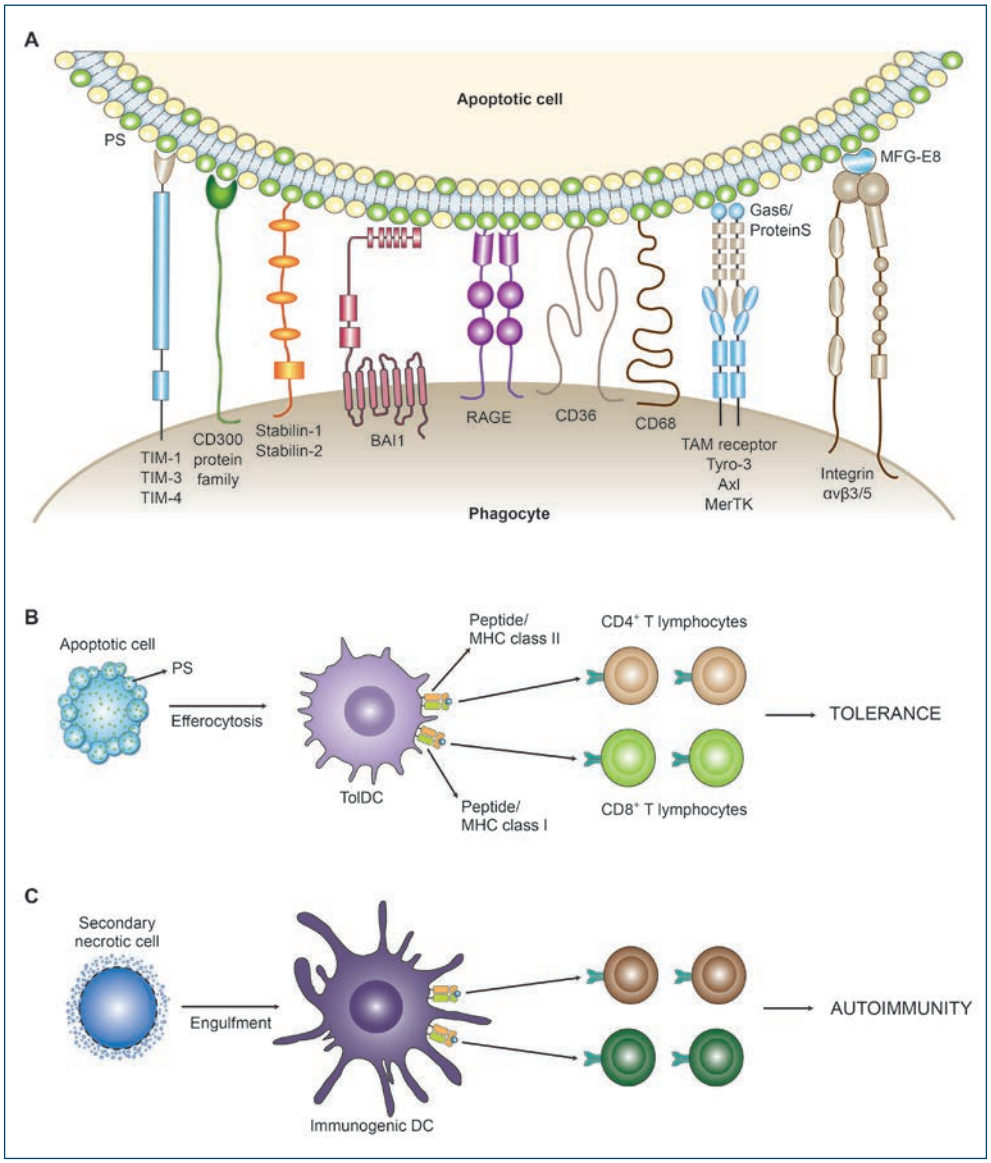


Figure 1. ***PS recognition, efferocytosis —or lack thereof— and its consequences.*** **(A)** *Phagocytes recognise apoptotic cells through PS receptors, which include those that can bind PS directly, such as TIM-1, TIM-3 and TIM-4, the CD300 protein family, Stabilin-1 and Stabilin-2, BAI1, RAGE and scavenger receptors like CD36 and CD68, and those that do so through complexes, like TAM receptors (Tyro-3, Axl, and MerTK)-Gas6/ProteinS and MFG-E8-αvβ3/5 integrin. Adapted from Elliott* et al.*, 2017 and Moller-Tank and Maury, 2014.* **(B)** *When undergoing apoptosis, the cell exposes PS molecules on its surface so that DCs can efferocyte them and present their autoantigens through MHC class I molecules to CD8⁺ T lymphocytes and through MHC class II molecules to CD4⁺ T lymphocytes in a tolerogenic manner.* **(C)** *Late apoptotic cells, namely secondary necrotic cells, lose their membrane integrity and release danger signals that prompt the immunogenic presentation of autoantigens to CD4⁺ and CD8⁺ T lymphocytes, potentially leading to autoimmunity. Infographic by VRB and SRF.*

1.2. Autoimmune disorders

As previously stated, autoimmune diseases are caused by autoimmune reactions that are severe enough to cause disease. Currently, more than 80 autoimmune diseases have been described (Luan *et al.*, 2017), and they reportedly affect up to 7-9% of the global population (Cooper *et al.*, 2009). With current medical interventions, most autoimmune diseases have become chronic, but they still entail debilitation, higher morbidity and mortality.

The primary cause of autoimmune diseases is the failure of central and peripheral tolerance mechanisms. In fact, two systemic autoimmune diseases —the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and the autoimmune polyendocrine syndrome type 1 (APS-1) syndrome—, caused by mutations in the *FoxP3* gene and the *AIRE* gene, respectively, reflect the vital importance of these two processes (Su and Anderson, 2009). Nonetheless, in most autoimmune diseases, the mutation of a single gene cannot explain the development of the disease, and these are known to be influenced by predisposing genetic factors —frequently shared among the different autoimmune disorders (Richard-Miceli and Criswell, 2012)—, environmental factors and stochastic factors. These multifactorial autoimmune diseases are classified depending on the targeted tissue. For example, the pathogenic role of autoantibodies causes systemic autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus, and also organ-specific autoimmune diseases like Addison's disease, Graves' disease and myasthenia gravis. In other organ-specific autoimmune illnesses, however, autoantibodies have a secondary role, and T cells are the ones to mediate the autoimmune destruction. That is the case of type 1 diabetes (T1D) and multiple sclerosis (MS), two paradigmatic T cell-mediated autoimmune conditions that target the pancreatic β-cells and the neuronal myelin sheaths, respectively. In the current scenario, both are chronic diseases that affect mostly young population and have an enormous impact on the quality of life of patients, accounting for high rates of disability, morbidity and mortality.

2. Type 1 diabetes

T1D, formerly known as juvenile-onset or insulin-dependent diabetes mellitus, is a chronic metabolic disease characterised by hyperglycaemia, which results from the destruction of insulin-producing pancreatic β-cells and subsequent insulin deficiency. The cause of this depletion divides T1D into two categories: type 1A and type 1B (ADA Position Statement, 2019). Considering type 1A diabetes, hyperglycaemia arises due to the selective autoimmune destruction of β-cells. This kind of diabetes is strongly associated with particular human leukocyte antigen (HLA) haplotypes and

usually manifests with autoantibodies against β-cell autoantigens. As for type 1B diabetes, the triggering factors leading to the destruction of β-cells are unknown, and the disease is not associated with specific HLA haplotypes nor autoimmunity. The present work will be solely based on type 1A diabetes, henceforth referred to as T1D.

2.1. Natural history of the disease

In T1D, the autoimmune reaction targets insulin-producing β-cells, which are found in the islets of Langerhans —the endocrine portion— of the pancreas. The pancreas is a gland organ located in the upper left side of the abdomen, surrounded by the stomach, the duodenum and the spleen. Morphologically, it is subdivided into a series of lobules separated by connective tissue. The adult pancreas is constituted by 95% of exocrine tissue, which is responsible for the production of pancreatic enzymes that aid digestion, and 5% of endocrine tissue, which plays a vital role in metabolic control. Regarding the exocrine portion, acinar cells are responsible for the production of digestion enzymes that are collected by a complex ductal network draining into the duodenum. The cells in the endocrine compartment are organised in small clusters called islets of Langerhans, which release the hormones secreted into the bloodstream. These islets contain five types of cells (Gartner and Hiatt, 2014), with most being either β-cells, which account for 75% of total islet cells and secrete insulin and amylin in response to high blood glucose concentration, or α-cells (20%), which produce glucagon in response to low glycaemia levels. Moreover, the islets are composed of δ-cells (4%), which release somatostatin to inhibit glucagon and insulin secretion and vasoactive intestinal peptide; PP cells (1%), which produce the pancreatic polypeptide hormone to regulate the activity of the endocrine and exocrine pancreas, ε-cells (<1%), which secrete ghrelin to inhibit insulin secretion, and G-cells (<1%), which secrete gastrin to stimulate the production of hydrochloric acid by parietal cells of the stomach.

Focusing on T1D, its onset echoes the severity of the destructive autoimmune process executed against β-cells. Currently, the definitive triggering factors causing the breach of tolerance are still elusive, but several genetic, environmental and stochastic factors reportedly contribute to disease origin (ADA Position Statement, 2019). T1D is mainly diagnosed at infancy and adolescence, following a commonly long asymptomatic period known as prediabetes. In the so-called stage 1 of T1D evolution (**Figure 2**), the leukocytic infiltrate (insulitis) progressively invades the islet and attacks β-cells. Although no signs or symptoms of the disease are present yet, this process is not entirely silent. For instance, autoantibodies against different β-cell autoantigens —insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma antigen-2 (IA-2) and zinc transporter 8 (Znt8)— appear sequentially in the sera during this period (Gorus *et al.*, 2013; Mayer-Davis *et al.*, 2018). They are considered the

earliest biomarkers of β-cell autoimmunity and serve to predict the speed of progression into T1D (Ziegler *et al.*, 2013). In fact, the presence of two or more autoantibodies in children is associated with a >80% risk of developing T1D by the age of 18 years. Notwithstanding, time from seroconversion to overt diabetes is variable, ranging between weeks to decades. Furthermore, since the β-cell mass is probably diminishing and endogenous insulin secretion might be unable to maintain normal glycaemia levels, an oral glucose tolerance test or a random fasting glucose test could appear altered (Regnell and Lernmark, 2017). The appearance of modest dysglycaemia marks the beginning of the stage 2 of T1D evolution.

When the decline in β-cell mass is such that endogenous insulin secretion can no longer meet metabolic demands, overt hyperglycaemia develops. Initially, the elimination of 80-90% of β-cell mass was deemed necessary to manifest pathological hyperglycaemia (Gepts, 1965), but recent data suggest that even lower percentages of destruction can cause diabetes (Pugliese, 2016). Upon reaching this stage, patients undergo several pathophysiological changes that ultimately explain the four main clinical signs by which T1D is diagnosed: polyuria, polydipsia, polyphagia and substantial weight loss. First, hyperglycaemia leads to a high concentration of glucose in urine (glycosuria) when the blood is filtered in the kidneys, resulting in an osmotic effect that entails excessive water loss and abundant production of urine (polyuria). This causes dehydration and, alongside excessive concentration of glucose in the blood, triggers excessive thirst mechanisms (polydipsia). Regarding polyphagia or excessive hunger, insulin has appetite-suppressing effects in the hypothalamus, and its absence makes so that appetite cannot be satiated. Finally, as muscles cannot absorb glucose to make up energy in the absence of insulin, lipolysis is induced in the liver to obtain this energy. The utilisation of fatty acids leads to substantial weight loss in a short amount of time, but this process also entails the production of ketone bodies. Although they can be excreted in urine, their continuous generation causes their accumulation in blood, which is known as ketoacidosis and is the most dangerous short-term complication of diabetes (Kahanovitz *et al.*, 2017).

The clinical diagnosis marks the beginning of stage 3 of T1D evolution (**Figure 2**). T1D can be diagnosed based solely on a fasting plasma glucose of >126 mg/dL or a random plasma glucose >200 mg/dL (ADA Position Statement, 2019). Nonetheless, the measurement of other biomarkers can provide additional information. For instance, glycated haemoglobin or haemoglobin A1c (HbA1c) is a widely-used biomarker that measures the glycation of haemoglobin and thus reflects average blood glucose levels over a three-month period. Thus, it can be used to diagnose diabetes (>6.5% or 48 mmol/mol) or to monitor blood glucose control. Moreover, in order to confirm that the absence of insulin is due to a selective autoimmune attack against β-cells, autoantibodies targeting β-cell autoantigens can be measured in the sera. It is worth mention-

ing that, currently, systematic screening for autoantibodies against β-cell autoantigens in general population or even in subjects at risk is not recommended for ethical reasons, given that no therapeutic interventions have demonstrated efficacy in preventing or reversing the disease (Aguilera, 2017). Also, the function of the residual pancreatic β-cell mass reservoir can be assessed through the measurement of basal and stimulated C-peptide levels. C-peptide is a 31-residue molecule that connects the chains A and B of the insulin molecule when proinsulin has not yet been processed to insulin. When the proteolysis occurs, equimolar amounts of C-peptide and insulin are stored in the secretory granules of β-cells, and are released together into the bloodstream (Wahren and Larsson, 2015). Since the measurement of insulin levels could be altered due to hepatic catabolisation and exogenous insulin administration, measurement C-peptide, which purely reflects the endogenous insulin secretion and escapes hepatic clearance, is more reliable than measuring insulin itself. Additionally, this biomarker can be tested in fasting conditions or after glucagon or mixed-meal stimulation, providing significant information about glucose tolerance dynamics. In patients with T1D, as there is little or no insulin secretion, plasma C-peptide levels are often found low or undetectable (ADA Position Statement, 2019). Finally, HLA alleles can be typed to determine if the subject has a predisposing HLA haplotype. Upon diagnosis, the individual will require exogenous insulin administration for life.

2.1.1. Available treatment

Exogenous insulin administration is the gold-standard treatment for patients with T1D. This hormone-replacement therapy is implemented with the aim to decrease and manage pathological hyperglycaemia. In 1920, Nobel Prize winner Dr Frederick Banting and Dr Charles Best discovered that pancreas extracts could ameliorate the clinical signs of diabetes and delay the untimely death of the children affected, which marked a crucial breakthrough in the history of T1D. A young Canadian patient named Leonard Thomson was the first human to receive an insulin injection only two years later (Karamitsos, 2011). Ever since, several pharmaceutical companies have invested significant efforts to improve the yield and purity of insulin, which started at insulin purification from pigs and cattle and have evolved into the recombinant production of human insulin (Vecchio *et al.*, 2018). Now, close to a century after its discovery, different regimens of short-acting and long-acting insulin analogues injected subcutaneously (s.c.) or continually-infused with the use of an insulin pump have transformed T1D into a manageable disease. Moreover, many different formulations of insulin analogues exist to suit the patient's needs (Aathira, 2014). The total daily insulin dose is based on the weight of the patient and ranges between 0.4 and 1.0 IU/kg/day (American Diabetes Association, 2019).

Although insulin treatment has allowed many patients to live on a relatively healthy life (The Diabetes Control and Complications Trial Research Group, 1993), it does not relieve the disease burden and only delays the appearance of long-term secondary complications. In fact, short-term complications of diabetes include recurrent hypoglycaemia episodes, hyperglycaemia with or without ketoacidosis, glycaemia excursions, poor wound healing and susceptibility to certain infections due to malfunctioning of the immune system (ADA Position Statement, 2019). The long-term secondary complications take decades to appear and include retinopathy with potential loss of vision, peripheral neuropathy with risk of foot ulcers and amputations, autonomic neuropathy causing genitourinary and cardiovascular diseases, and nephropathy leading to renal failure (Melendez-Ramirez *et al.*, 2010). In the end, this myriad of secondary complications causes distressing morbidity and mortality rates in patients with T1D. As a whole, diabetes represents a vast economic expense on the national health system and a significant life-altering circumstance for patients, and so T1D has emerged as an urgent biomedical matter in every sense.

Additionally, albeit at an overly slow pace, many other β-cell replacement strategies are finding their way into the clinics, such as artificial pancreas technology accompanied by continuous glucose monitoring systems (Boughton and Hovorka, 2019), islet transplantation, stem cell therapy and β-cell reprogramming, which could benefit from encapsulation devices for protection against the autoimmune response (Peloso *et al.*, 2018).

2.1.2. Partial remission

Shortly after the diagnosis and the initiation of insulin therapy, 80% of patients experience a short period when near-normal metabolic control is maintained while insulin requirements decline by 50%. This period is known as partial remission phase (PR) or honeymoon, and it is spontaneous and transitory (Fonolleda *et al.*, 2017). There is no consensus in the clinical definition of the PR, but experts agree that insulin requirements must be <0.5 International Units (IU)/kg of body weight/day and HbA1c <7%. Recently, both parameters have been combined in a new formula named *insulin dose-adjusted HbA1c* (IDDAA1c), which is defined as HbA1c (%) + 4 × (insulin dose in IU/kg/24 hours) and suggests that a value <9 would indicate PR (Kordonouri *et al.*, 2014). Although definitive causes are not understood, experts believe that this phase reflects an attempt at islet regeneration and improvement of β-cell function in a putative transient recovery of adaptive tolerance to β-cells (Fonolleda *et al.*, 2017). Indeed, both metabolic and immune factors are thought to contribute to its development. Specifically, β-cells could be relieved from the stress of controlling glycaemia after the start of insulin therapy, which could thwart the aggressiveness of

the autoimmune attack and allow for an increase in their replication and better function. However, this apparent improvement in β-cell function could eventually promote the release of autoantigens to the extracellular media, which would re-activate the autoimmune attack towards these newly-generated or functionally-improved β-cells, thus perpetuating a vicious circle of regeneration and autoimmunity. After this short period of PR, patients become once again dependent on exogenous insulin administration to survive and enter stage 4 of T1D evolution (**Figure 2**).

2.2. *Epidemiology*

T1D is one of the commonest metabolic diseases in childhood. T1D represents 5-10% of the total cases of diabetes, as compared to the greater part of adult patients with diabetes who suffer from type 2 diabetes (T2D). T1D incidence has a noted geographical variability, with countries in Central and Eastern Europe and North America displaying the highest incidences, and countries in Asia and Africa showing the lowest (International Diabetes Federation, 2017). In Europe, the distribution of T1D incidence displays a north-south gradient (Borchers *et al.*, 2010), with the highest rates of incidence found in northern countries such as Finland

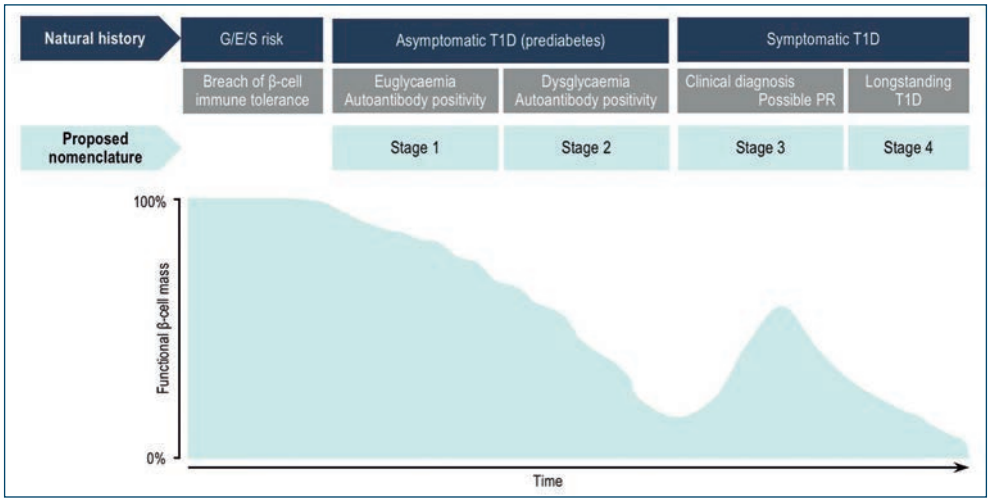


Figure 2. ***Proposed staging model of T1D.*** *In individuals genetically at risk, after exposure to specific environmental triggering factors and occurrence of specific stochastic factors (G/E/S risk), tolerance to β-cells is breached. Afterwards, immune tolerance checkpoints are bypassed and the immune response targets β-cells. During prediabetes, stage 1 marks the start of this process, with individuals having two or more autoantibodies directed to β-cell autoantigens and healthy blood glucose concentration. In stage 2, individuals show dysglycaemia without severe symptoms. Stage 3 begins at clinical diagnosis, which can be followed by a transitory period of PR. Finally, stage 4 represents longstanding T1D. Adapted from Fonolleda* et al.*, 2017 and Regnell and Lernmark, 2017; infographic by VRB and SRF.*

(57.2 cases/100,000 inhabitants/year), Sweden (39.5 cases/100,000 inhabitants/year) and Norway (29.8 cases/100,000 inhabitants/year) (International Diabetes Federation, 2017). In Spain, recent epidemiology studies indicate that the incidence of T1D in children younger than 14 years of age is 14.3-23.2 cases per 100,000 inhabitants per year (Castell, 2017). Additionally, the global incidence of T1D is increasing by a dramatic 3-4% each year (Mayer-Davis *et al.*, 2017; Patterson *et al.*, 2019), most notably in children and adolescents. With around 100,000 newly diagnosed cases each year (International Diabetes Federation, 2017), worldwide T1D prevalence is estimated at 2-3 cases per 1,000 inhabitants (Castell, 2017).

The onset frequently occurs in childhood, its incidence peaks being at 5-9 and 10-14 years of age. Nowadays, however, its incidence is seemingly levelling off across all age ranges, thus also affecting children below 4 years (Borchers *et al.*, 2010), young adults (Lipman *et al.*, 2013) and even adults (Bruno *et al.*, 2016). Finally, broadly speaking, T1D does not exhibit a gender bias, although this issue remains controversial (Diaz-Valencia *et al.*, 2015).

2.3. Aetiology

Currently, the exact aetiology of T1D remains unknown, although several genetic, environmental and stochastic factors are thought to precipitate its development. In fact, disease concordance between relatives —higher than 50% in monozygotic twins, 5-10% in non-twin siblings— points at the contribution of non-genetic factors in disease development. Also, the risk for children who have a parent with T1D is 1-9%, as compared to the 0.5% risk of the general population (Harjutsalo *et al.*, 2005; Pugliese *et al.*, 2016; Redondo *et al.*, 2008; Steck and Rewers, 2011; Triolo *et al.*, 2019). In the last decade, one macro-study has made significant contributions in finding new genetic and environmental triggers for T1D and confirming or discarding the role of those previously acknowledged. The Environmental Determinants of Diabetes in the Young study (TEDDY, teddy.epi.usf.edu) has recruited and prospectively followed children from USA, Germany, Sweden and Finland for 15 years to identify genetic-environmental interactions, infectious agents, dietary and other environmental and lifestyle factors that could trigger or protect against T1D in genetically susceptible individuals (Hagopian *et al.*, 2006).

2.3.1. Genetic factors

T1D is the perfect example of polygenic disease, with more than 50 loci conferring susceptibility in humans (Rich *et al.*, 2009). They have been defined as insulin-dependent diabetes mellitus (IDDM) loci, and further information on them can be found

in the T1Dbase database (www.t1dbase.org). Current genetic data indicate that polymorphisms in **HLA genes** (IDDM1), which are located in chromosome 6p21, confer 50% of the familial genetic risk (Regnell and Lernmark, 2017). Among them, genes coding for HLA class II molecules have been strongly associated with the disease. Particular combinations of HLA-DRB1, HLA-DQA1 and HLA-DQB1 alleles, such as haplotypes HLA-DRB1*0301-DQA1*0501-DQB1*0201 (DR3) and HLA-DRB1*0401-DQA1*0301-DQB1*0301 (DR4-DQ8) (Krischer *et al.*, 2015), are known to increase the risk of T1D, whereas haplotypes like HLA-DRB1*1501-DQA1*0102-DQB1-0602 (DR15-DQ6) are reported to protect against it (DiMeglio *et al.*, 2018). In fact, the DR3 haplotype is present in 34% of individuals with T1D (Erlich *et al.*, 2008), and Carlsson *et al.*, 2012 reported that 89% of children with T1D of all 3,500 included in their study had at least one copy of DQ8 and DQ2 haplotypes. The HLA class I region has been found to influence T1D development as well, although its contribution is reportedly smaller. For instance, the HLA-B*3906 allele has been widely associated with T1D risk, and the HLA-A*02 allele increases the risk of development in those individuals with DR3/4-DQ8 haplotypes (Noble and Erlich, 2012). By contrast, HLA-B*5701 allele protects against T1D development (Noble *et al.*, 2010). Experts argue that polymorphisms in HLA class I alleles might affect the age of onset rather than the absolute risk of disease (Noble and Erlich, 2012).

Genome-wide association studies (GWAS) have identified **non-HLA genes** also conferring susceptibility, although less prominently. Many of them are implicated in immune system regulation and β-cell function and highlight pathways that are important in disease development, such as the regulation of T lymphocytes activation, the response against viral infections and the expression of autoantigens (Nyaga *et al.*, 2018; Sosenko *et al.*, 2017). Among them, polymorphisms in the *INS* gene (IDDM2) and its promoter regions have shown the strongest association with disease development (Pociot *et al.*, 2010). Interestingly, the polymorphic Variable Number of Tandem Repeats (VNTR) of the *INS* gene determines the expression of insulin in human TEC (Cai *et al.*, 2011; Sparks *et al.*, 2016), which is 200-300% higher for IDDM2 alleles associated with resistance to T1D development than for those alleles acknowledged as predisposing (Pugliese *et al.*, 1997), thus impacting on the efficiency of thymic selection (Pugliese, 2004). The immune-related *PTPN22*, which codes for the tyrosine phosphatase protein LYP, also has a significant contribution. In fact, this protein is a negative regulator of T cell receptor signalling, and so specific mutations could aid in autoreactive T cell survival (Cerosaletti and Buckner, 2012). Other allelic variations associated with T1D have been found in the islet-specific glucose-6-phosphatase catalytic subunit-related protein (*IGRP*), *IL2RA* (IDDM10), *CTLA4* (IDDM12), *IFIH1* (IDDM19), *PTPN2*, *SH2B3*, *PDHB*, *PPIL-2* and *IL10* genes (Pociot and Lernmark, 2016; Sharma *et al.*, 2018). Interestingly, the TEDDY study

has revealed that specific mutations of genes associated with the disease can influence the specificity of the first appearing autoantibody (Törn *et al.*, 2015).

Additionally, gene expression can be widely affected by environmental factors through **epigenetic mechanisms**, such as DNA methylation and histone acetylation (Jerram *et al.*, 2017). Studies in human pancreatic islets reported epigenetic changes in several loci known to confer risk of T1D that influence insulin secretion, apoptosis and proliferation of β-cells (Olsson *et al.*, 2014). Similarly, altered histone methylation patterns in key immune-function genes have been found in lymphocytes from patients with T1D (Miao *et al.*, 2008). In the future, the combined analysis of HLA and non-HLA loci and epigenetic modifications could aid in predicting the risk of developing T1D.

2.3.2. *Environmental factors*

The sharp increase in T1D incidence observed in the last decades cannot be explained only by genetic factors, since the gene pool has been stable over this period, and so this suggests that the impact of environmental factors and lifestyle must be substantial. Several epidemiological studies have proven that **migrants** show an incidence of T1D that is similar to that of their host population (Bodansky *et al.*, 1992; Oilinki *et al.*, 2012; Söderström *et al.*, 2012). Also, the risk of T1D differs substantially in neighbouring populations who live in **different socioeconomic conditions**. The paradigmatic example is the Karelian Republic of Russia and Finland, which are genetically similar but far apart in terms of T1D incidence, with Finland having also greater incidence rates of coeliac disease, autoimmune thyroid disease and allergy (Kondrashova *et al.*, 2013). Other studies have reported higher incidence rates of T1D in urban areas when compared to rural areas as well (Allen *et al.*, 1986; Miller *et al.*, 2011). A possible explanation for this phenomenon is the **hygiene hypothesis**, which suggests that the developing immune system needs stimulation by pathogenic environmental factors to achieve an adequate maturation (Kolb and Elliott, 1994). Indeed, industrialised countries have reduced infectious diseases rates by using antibiotics, vaccines and better hygiene. Although this has undoubtedly prevented unnumbered diseases and deaths, this transformation could also deter the proper maturation of the immune system and lead to defects in the establishment of immune tolerance, thus contributing to the higher incidence of autoimmune diseases and allergies in developed countries (Bach and Chatenoud, 2012).

On the opposite side of the spectrum, a wide range of **microbial agents** has been involved in the development of T1D in those individuals genetically at risk. These pathogens could cause the disease by infecting the β-cell and prompting direct cytotoxicity, but other proposed mechanisms include molecular mimicry or cross-reactivity between islet autoantigens and microorganisms' antigens, or the release of β-cell

autoantigens to nearby inflammatory microenvironment (Principi *et al.*, 2017). Many studies have revealed that several enteroviruses, rubella virus, coxsackievirus and rotavirus infections are associated with T1D development (Coppieters *et al.*, 2012a). Moreover, respiratory infections such as common cold, influenza-like illness, sinusitis and laryngitis/tracheitis have also shown to correlate with risk of autoimmunity (Lönnrot *et al.*, 2017). To date, however, no definitive causal role has been established.

Given that the incidence of T1D is higher in northern regions of Europe and the United States, **low sun exposure** and **low vitamin D concentrations** have been proposed as environmental triggers for T1D. In fact, several studies have reported low levels of vitamin D in patients with T1D in comparison to healthy controls (Butalia *et al.*, 2016; Raab *et al.*, 2014), and studies in other autoimmune diseases have shown similar results (Rak and Bronkowska, 2018). Experts argue that the active form of vitamin D (calcitriol or 1,25-dihydroxycholecalciferol) can suppress the production of pro-inflammatory cytokines and favour the induction of Treg cells. Also, specific allelic variants of genes involved in vitamin D metabolism have been associated with increased risk of T1D. Moreover, a **seasonal pattern** has been reported for T1D onset in most countries, with its incidence being higher during the coldest months and lower during the warmest ones (Moltchanova *et al.*, 2009). Overall, these findings suggest that vitamin D is a potent immunomodulator that could protect β-cells from the autoimmune attack.

Different studies suggest that the composition and function of the **human microbiome**, increased permeability of the intestinal barrier and altered gut mucosal immune response are also candidate environmental factors influencing T1D development (Butalia *et al.*, 2016; Stewart *et al.*, 2018). Children with T1D have shown reduced diversity in their microbiome (De Goffau *et al.*, 2013; Kostic *et al.*, 2015) and subclinical inflammation of the gut (Vaarala, 2013), pointing to the significant immunomodulatory effect of the intestinal microbiome (Needell and Zipris, 2016). Closely related to the microbiome, **diet** is another environmental factor that could be involved in the aetiology of the disease. Several findings suggest that breastfeeding at the time of introduction of gluten and solid foods might contribute to protection against T1D (Jackson and Nazar, 2006), whereas early introduction of cow's milk has been reported as a risk factor (Lamb *et al.*, 2015). Although the acceptance of the connection between cow's milk consumption and T1D development remains controversial, proposed mechanisms include cross-reactivity between β-casein protein present in cow's milk and glucose transporter 2 (GLUT-2) expressed on β-cells, or the influence of insulin in cow's milk-based formulas on insulin autoantibody formation (Chia *et al.*, 2017). Moreover, patients with newly-diagnosed T1D have shown increased T cell reactivity against gluten (Klemetti *et al.*, 1998) and presence of anti-gliadin antibodies in their serum (Catassi *et al.*, 1987), hinting at the early consumption of gluten as a risk factor for T1D as well.

Prenatal and perinatal factors have attracted special attention in the pathogenesis of T1D. Aside from the significantly higher T1D concordance in dizygotic twins in comparison to non-twin siblings, which points to a relevant effect of the *in utero* environment, increased age of the mother has been associated with an increased incidence of T1D (Stene and Gale, 2013). Logically, medical interventions during this period could alter the immune response in adult life, and this is exemplified by the association of antenatal corticosteroid exposure and the increased incidence of T1D in this offspring (Greene *et al.*, 2013). Moreover, infants who develop T1D later in life possess a subset of β-cell autoreactive $CD4^+$ T cells with an unusual pro-inflammatory gene signature [pre-T helper (Th)1/Th17/T follicular helper (Tfh) cell], which could be the result of early priming by specific perinatal factors (Heninger *et al.*, 2017).

Finally, **deeply stressful events** could contribute to the development of T1D. In Nygren *et al.*, 2015, researchers found that the children having experienced a serious life event —such as the death of a relative, a new family structure or conflict at home— had three times higher risk of developing T1D. A proposed mechanism to explain the connection between stress and T1D development is that serious life events could activate the physiological stress response and release of cortisol, thus increasing insulin resistance and, in turn, insulin demands, ultimately contributing to **β-cell stress**. Furthermore, if present β-cells and insulin release were not able to meet them, the resulting increase in glycaemia would promote a glucose toxicity environment in the pancreas, further contributing to β-cell death (Rewers and Ludvigsson, 2016). In this sense, several situations that increase the body's requirement of insulin could potentially be risk factors for the development of the disease, and these include rapid longitudinal growth, trauma, low physical activity and consequent overweight and obesity, infections and puberty (Regnell and Lernmark, 2017).

Taken together, even if many factors have been postulated as potential environmental triggers for T1D, these data point to the combination of factors as being crucial in the development of the disease in individuals genetically at risk.

2.3.3. Stochastic factors

Genetically identical twins, even when exposed to the same environment, show differences in the development of T1D. That is why stochastic factors and, among them, the **random rearrangement of the TCR and BCR genes**, are believed to play a relevant role in T1D pathogenesis (Vives-Pi, 2017). As previously discussed, the proper functioning —or malfunctioning— of several control checkpoints present in **central and peripheral tolerance mechanisms** could also be decisive in the triggering of β-cell autoimmunity or the progression to clinical onset.

2.4. Pathological process in human T1D

After the breach of β-cell tolerance, a mild leukocytic infiltrate starts surrounding the pancreatic islets and advances gradually through the cellular clusters until reaching β-cells in the islets of Langerhans (Planas *et al.*, 2010). Since the pancreas is a hardly accessible organ, data on the pathogenic role of the immune system in T1D have been largely obtained from findings described in peripheral blood of the patients and pancreas necropsies and donations from prediabetic subjects and patients with T1D who died at different stages of the disease. Pancreatic biopsies are not usually taken in patients with T1D due to the high risk of pancreatitis that is inherent to the procedure. In the last decades, however, the Network for Pancreatic Organ Donors with Diabetes (nPOD) (jdrfnpod.org) has procured pancreata and related tissues (lymph nodes, spleen, thymus, bone marrow and peripheral blood) from deceased organ donors with the disease and subjects positive for β-cell autoantibodies to laboratories around the world, allowing scientists to obtain valuable data on the histopathology and immunopathology of T1D and to challenge longstanding dogmas (Kaddis *et al.*, 2015). The other significant contributors to the expanding knowledge in the field have been experimental models. Animal models of disease are employed to investigate human diseases from a preclinical point of view, provided that their disease is sufficiently similar to the one suffered by humans. Through the years, they have constituted invaluable tools to achieve a deeper understanding of pathological processes and have allowed for the testing of select drug or therapy candidates prior to clinical trials. With the use of experimental models of disease in research, it is mandatory that ethical questions are addressed and guidelines for their care are followed strictly.

2.4.1. The role of the immune system

The main feature of the targeted organ in T1D is the leukocytic, albeit predominantly lymphocytic, infiltrate that invades the islets of Langerhans, which is responsible for the loss of β-cell mass and reduced endogenous insulin production. This phenomenon, termed **insulitis**, was firstly described in the early 20th century in a 10 year old patient who died of ketoacidosis (Schmidt and MB., 1902), and later on reported as a characteristic of the disease (Gepts, 1965). In the pancreas of patients with T1D, four broad categories of islet are present at the same time, which points to the progressive nature of the autoimmune attack: 1) normal islets without evidence of inflammation; 2) islets encircled by insulitis with a healthy number of β-cells per islet (early-stage insulitis); 3) islets surrounded and invaded by insulitis with a reduced number of β-cells (late-stage insulitis); and 4) islets taken over by insulitis and devoid of β-cells (end-stage insulitis) (Foulis *et al.*, 1986). It is worth noting that other endo-

crine cell types do survive the autoimmune attack, although the exocrine acinar cells surrounding the islets can be invaded by different immune cell subtypes and appear atrophied (Foulis and Stewart, 1984; Rodriguez-Calvo *et al.*, 2014). This could provide an explanation for the reduction in pancreas weight and volume that is observed in patients with T1D (Campbell-Thompson *et al.*, 2015). Nonetheless, the fraction of infiltrated islets in the human pancreas is generally low, ranging between 10 and 30% (Pugliese, 2016). Also, due to the lobular morphology of the pancreas, insulitic islets have been found clustered together within individual lobules, while other lobules appear completely unaffected (Richardson *et al.*, 2011).

Within infiltrated human islets, the most prominent cells found are $CD8^+$ T lymphocytes (Planas *et al.*, 2010; Willcox *et al.*, 2009). They could participate in β-cell destruction due to their ability to recognise antigenic targets in the context of MHC class I and destroy β-cells through the perforin-granzyme and Fas pathways (Graham *et al.*, 2012). Together with $CD68^+$ macrophages, $CD4^+$ T lymphocytes, B lymphocytes and DCs, they are the main effectors of β-cell destruction (**Figure 3**). Treg cells, plasma cells, neutrophils, mast cells and Natural Killer (NK) cells rarely appear (Morgan and Richardson, 2018; Willcox *et al.*, 2009), even though Dotta *et al.*, 2007 described the presence of a significant number of NK cells in inflamed islets of recent-onset patients with T1D.

Regarding the pathogenic role of T cells in T1D, autoreactive **$CD4^+$** and **$CD8^+$ T cells** have been found circulating in patients with T1D and, unlike the ones present in control subjects, they are markedly enriched in memory phenotype, meaning that they have encountered their antigen and proliferated in response to that stimulation (Monti *et al.*, 2007, 2009; Öling *et al.*, 2012). Moreover, defects in **Treg cells** have been described in patients with T1D (Hull *et al.*, 2017). For instance, several genes influencing the risk of T1D do have a role in the fitness and function of $FoxP3^+$ Treg cells, such as the *IL2*, *IL2RA*, *PTPN2*, *PTPN22*, *CTLA4* and *IL10* genes (Todd *et al.*, 2007), and thus it is plausible that the improper function of Treg cells is a contributing factor in T1D (Hull *et al.*, 2017). In fact, Treg cells of patients with T1D reportedly have higher pro-inflammatory cytokine profile (McClymont *et al.*, 2011), lower ability to control autologous T effector proliferation (Lindley *et al.*, 2005; Schneider *et al.*, 2008), increased levels of apoptosis (Glisic and Jailwala, 2012), decreased stability of FoxP3 expression (Long *et al.*, 2010) and different microRNA (miRNA) signature (Hezova *et al.*, 2010). Recent studies have also pointed at the fact that $FoxP3^+$ Treg cells could not be one defined population but a heterogeneous mixture of cellular subtypes that employ different methods to regulate the immune response (Mason *et al.*, 2015). A decreased frequency of activated Treg cells ($CD4^+CD25^+FoxP3^+CD45RO^+$) has been described in patients with T1D, and the authors proved this defect was associated with a trend for lower residual C-peptide levels and poorer metabolic control

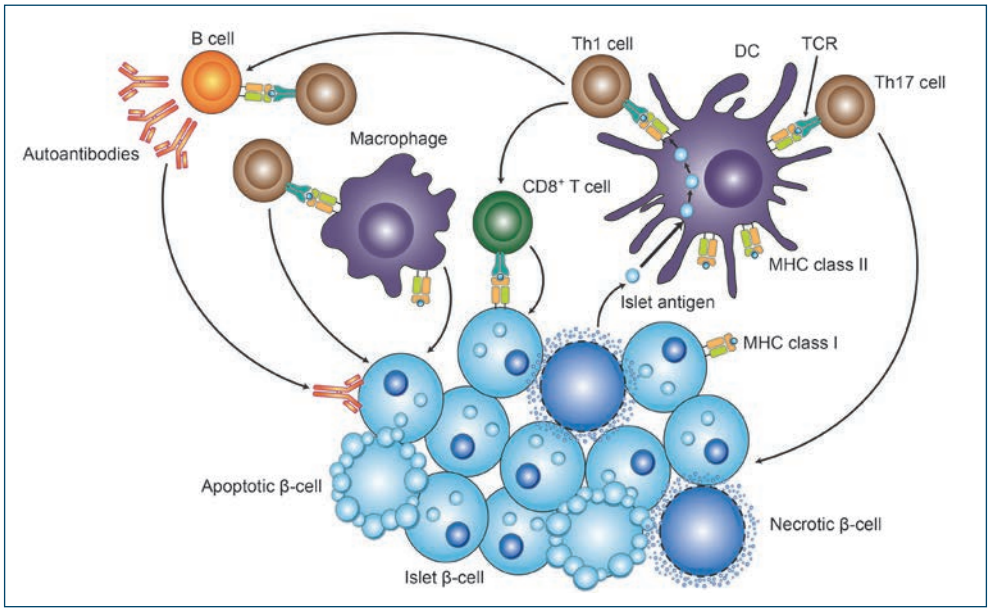


Figure 3. ***CD8⁺ T lymphocytes, CD4⁺ T lymphocytes, DCs, macrophages and B cells are the main cellular effectors of the pathological process that leads to β-cell destruction in T1D.*** *Pancreatic β-cells release antigens, which are captured by APCs like DCs and macrophages. They process these proteins and mount an immune response that involves Th1 and Th17 T cells and cytotoxic CD8⁺ T cells. Also, B cells, aside from secreting autoantibodies, could act as APCs to CD4⁺ T cells. Adapted from Roep and Peakman, 2010; infographic by VRB and SRF.*

(Okubo *et al.*, 2016). Additionally, islet-specific IL-10 secreting cells —putative **TR1 cells**— have been associated with a less aggressive form of T1D, since individuals who have higher functionality of these cells have exhibited lower levels of autoantibodies and pro-inflammatory islet-specific T cells (Arif *et al.*, 2014), a later age at clinical diagnosis (Arif *et al.*, 2004) and better glycaemic control (Sanda *et al.*, 2008).

B cells are also believed to contribute to T1D. Although the role of autoantibodies directed to β-cell antigens remains to be defined, their role as APCs could be prominent in driving the disease (Hinman and Cambier, 2014). In this sense, B cells from patients with T1D are defective in IL-10 production in response to toll-like receptor (TLR) stimulation (Jagannathan *et al.*, 2010), and patients show a low frequency of $CD19^{+}CD5^{+}CD1^{hi}$ cells circulating in peripheral blood, which are thought to be IL-10 producing regulatory B cells (Deng *et al.*, 2016). Interestingly, the differing proportion of **$CD20^{+}$ B cells** in islets of paediatric patients has been raised as evidence to the existence of two different endotypes within T1D. In children aged below seven years, $CD20^{+}$ cells are present in relative abundance, but their proportion is much lower in those patients who are older at clinical diagnosis (Leete *et al.*, 2016). This further supports the potential role that B cells have as APCs in the islets

(Graham *et al.*, 2012). Perhaps in accordance with the fact that histological differences point to several T1D endotypes, individuals diagnosed before seven years of age show around 80% of insulin-devoid infiltrated islets, whereas patients diagnosed at age 13 exhibit only 25% (Morgan, 2017). Importantly, the two endotypes seem to be reflected in peripheral blood (Arif *et al.*, 2014). The authors proved that patients diagnosed at age 6-10 years show higher frequency of multiple autoantibodies and predominantly interferon (IFN)-γ T cell responses against several autoantigens, whilst patients with clinical diagnosis at 18 years have lower frequency of multiple autoantibodies and respond with IL-10 secretion to the same stimulation. These pieces of evidence support the existence of a correlation between the earlier age at T1D diagnosis and the more aggressive form of the disease.

In T1D, both T cells and B cells direct their response against specific **autoantigens** (**Table 1**), but which fall into the "primary autoantigen" category and which are targets for bystander autoimmune attack is currently undefined (Arvan *et al.*, 2012). These primary autoantigens are considered the initial drivers of the disease, and it is accepted that they are processed by APCs and presented to naïve $CD4^+$ T cells in draining pancreatic lymph nodes (PLN). Upon activation, they secrete pro-inflammatory cytokines and activate β-cell-specific cytotoxic $CD8^+$ T cells, which are then recruited to the islets to produce cytokines and further stimulate the autoimmune attack against β-cells. Direct evidence of the importance of each autoantigen is practically impossible to obtain in humans, but indirect proof does exist, such as the detection of autoreactive $CD4^+$ and $CD8^+$ T lymphocytes and memory $CD4^+$ T cells in peripheral blood of patients, and active T cell responses prompted by autoantigen exposure, including proliferation and cytokine secretion (Han *et al.*, 2013). Furthermore, patients with T1D show high frequencies of autoantibodies directed against several of these autoantigens in their sera. Current evidence does not provide autoantibodies with an evident pathogenic role in the disease, and so they are used as biomarkers in two clinical settings (Atkinson, 2012): 1) to discriminate the autoimmune origin of the absence of insulin, and 2) to predict the onset of T1D in subjects at high genetic risk and in first-degree relatives of patients. Moreover, the sequential appearance of autoantibodies in the sera might reflect the increasing diversification of autoantigen specificities, a phenomenon that is known as *epitope spreading*. Briefly, the immunogenic response against an epitope would cause the destruction of the β-cell and the release of new proteins, which, alongside the pro-inflammatory environment, would elicit the activation of additional T cells with different islet antigen specificities (Roep and Peakman, 2012).

Apart from existent autoantigens, recent evidence suggests that inflammation and stress are plausible factors driving the generation of **neoepitopes**, which are unlikely available for negative thymic selection (James *et al.*, 2018). Many of these new antigens appear due to post-translational modifications (Pugliese, 2017), such

Table 1. List of the main autoantigens targeted by the adaptive immune system in human T1D

Autoantigen	Tissue distribution	Function	Autoantibodies	Autoreactive T cells
Preproinsulin/ Proinsulin/Insulin	β-cells, thymus	Glucose homeostasis	Yes	$CD4^+$, $CD8^+$
Amylin	β-cells	Glycaemic regulation	No	$CD8^+$
IGRP	β-cells	Glucose metabolism	No	$CD8^+$
ZnT8	β-cells	Zinc uptake in β-cell secretory granules	Yes	$CD8^+$
GAD65	β-cells, adrenal gland, central nervous system, neurons, testis, ovary	GABA synthesis	Yes	$CD4^+$, $CD8^+$
GAD67	β-cells (weakly), neurons	GABA synthesis	Yes	$CD4^+$
IA-2	β-cells	Regulation of hormone secretion	Yes	$CD4^+$, $CD8^+$
IA-2β	β-cells	Regulation of hormone secretion	Yes	$CD4^+$
ICA-69	Islet cells, heart, brain	Regulation of hormone secretion	Yes	$CD4^+$
Imogen 38	Islet cells	Granule metabolism	Yes	Yes
PDX1	β-cells	β-cell differentiation Insulin secretion	Yes	Not reported
Carboxypeptidase H/E	β-cells, neuroendocrine cells, adrenal glands	Proinsulin to insulin processing	Yes	Not reported
HSP60	Ubiquitous (mitochondria)	Protein folding in mitochondria	Yes	$CD4^+$
Sulfatide	Ubiquitous	Insulin secretion	Yes	Not reported
Ganglioside GM2-1	Islet cells	Metabolic suppression	Yes	Not reported

IGRP: glucose-6-phosphatase catalytic subunit-related protein; ZnT8: zinc transporter 8, GAD: glutamic acid decarboxylase; IA-2: insulinoma-antigen 2; ICA-69: islet cell antigen-69; PDX1: pancreatic duodenal homeobox protein 1; HSP60: heat shock protein 60. Adapted from Roep and Peakman, 2012 and Lampasona and Liberati, 2016.

as alternative splicing, citrullination, or deamidation, and predisposing HLA variants are indeed critical for the presentation of these neoepitopes to T cells (Doyle and Mamula, 2012). Moreover, other mechanisms such as different MHC binding registers, peptide fusion, destructive and constructive proteolysis and aberrant protein translation have also considerably enlarged the neoantigenic landscape of T1D. Interestingly, memory T cells reacting to these modifications have been detected at higher frequencies in patients with T1D than in controls, and patients often react to these cognate antigens with a pro-inflammatory IFN-γ response whereas controls respond with an IL-10 profile (Van Lummel *et al.*, 2014).

Professional APCs also contribute to T1D pathogenesis. CD11c$^+$ **DCs** present in the islet could help in the extravasation of autoreactive lymphocytes to the islets and drive *in situ* re-stimulation of these T cells. Moreover, **macrophages**, which are the second-most-frequent immune cell subset found in inflamed islets (Willcox *et al.*, 2009), could promote β-cell apoptosis by the secretion of pro-inflammatory cytokines (Uno *et al.*, 2007). Defects in phagocytosis processes have been described in humans suffering from T1D and autoimmune experimental models (Geerlings and Hoepelman, 1999; O'Brien *et al.*, 2006), meaning that these apoptotic β-cells would not be efficiently removed and could potentially transform into secondary necrotic cells, further contributing to inflammation. Supporting this, underlying immunological defects have been found in APCs obtained from patients with T1D (Creusot *et al.*, 2018). These include their development and numbers circulating in peripheral blood, antigen presentation, maturation, costimulation, cytokine production and tolerogenic functionality. Specifically, polymorphisms in the *FCGR2A* gene, which is involved in antigen capture and phagocytosis, and in the *ERAP1*, *CTSB* and *CTSH* genes, related to antigen processing, have been associated to T1D development (Alizadeh *et al.*, 2007; Evangelou *et al.*, 2014; Fung *et al.*, 2009). Also, patients with T1D show hyperactivation of the NF-κB pathway in monocytes and monocyte-derived DCs, and genes impacting on this pathway, such as the negative regulator *TNFAIP3* gene, have been associated to T1D (Barrett *et al.*, 2009). As for costimulatory molecules, many studies have reported differing results on the expression of CD40, CD80 and CD86 molecules on peripheral blood DCs and monocyte-derived DCs (Creusot *et al.*, 2018). Likewise, the measurement of the secretion of pro-inflammatory and anti-inflammatory cytokines by DCs has yielded different results, and so no definitive conclusions should be drawn in this regard. Nevertheless, certain polymorphisms in genes encoding for IL-10, IL-27 and IL-12 cytokine production have been associated with T1D (Creusot *et al.*, 2018). Regarding their tolerogenic functionality, peripheral blood APCs from patients with T1D are reportedly defective in FoxP3$^+$ Treg cells induction (Jin *et al.*, 2009).

Abnormally high levels of **cytokines** and **chemokines** may also play a role in the pathogenesis of T1D. Patients have shown parallel elevations of both pro-inflammatory and anti-inflammatory cytokines —IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-6, IL-12, TNF-α and granulocyte-macrophage-colony-stimulating factor (GM-CSF), among others— in peripheral blood when compared to control subjects, and their peripheral blood mononuclear cells (PBMCs) show higher levels of pro-inflammatory cytokine transcription —IFN-γ, TNF-α and IL-23— and secretion —IL-1α and TNF-α— upon stimulation (Chatzigeorgiou *et al.*, 2010; Fatima *et al.*, 2016; Gouda *et al.*, 2018; He *et al.*, 2014). While Th1 cells were once believed to be the sole pathogenic mediators in T1D, further dissecting studies have shown that patients with T1D have increased concentrations of IL-17 detectable in their sera concomitant

with higher frequencies of Th17-phenotype $CD4^+$ T cells, which do respond with IL-17 secretion to β-cell autoantigen stimulation as well (Arif *et al.*, 2011; Ferraro *et al.*, 2011; Marwaha *et al.*, 2010). Accordingly, IL-23 has also been detected in higher levels in the sera of patients (Fatima *et al.*, 2016), further pointing to the role of the Th17 pathway in T1D pathogenesis (Solt and Burris, 2015). Another critical question is how immune cells arrive at the islets. It is accepted that infiltrated islets secrete chemokines, which call for several immune cell subtypes to migrate to the source. For instance, CXCL10 is one of the most frequently detected chemokines in inflamed islets, and it interacts with its cognate receptor CXCR3, expressed by subsets of autoreactive $CD3^+$ cells (Roep *et al.*, 2010). Other chemokines, such as CCL2, CCL5, CCL8, CXCL1, CXCL2, CXCL9 and CX3CL1 can be detected in human islets, albeit at lower levels, and they chemoattract macrophages, T lymphocytes and neutrophils to further exacerbate insulitis (Burke and Jason Collier, 2015; Sarkar *et al.*, 2012). Interestingly, high levels of type I IFNs have also been detected in pancreatic islets and in the sera of patients, a fact that could support a viral involvement in T1D pathogenesis (Chehadeh *et al.*, 2000; Huang *et al.*, 1995).

Logically, most of the immunological defects listed in this section have been found in patients who already had overt diabetes, and so the effect that **hyperglycaemia** and **glycaemia imbalance** might have in the immune system cannot be underestimated. Importantly, several studies have demonstrated that better glycaemia control can improve the function of polymorphonuclear cells, monocytes, macrophages, T lymphocytes, tolDCs and Treg cells (Dáňová *et al.*, 2017; Geerlings and Hoepelman, 1999), and even macrophage function and phagocytosis in patients with T2D has been enhanced with a better metabolic control (Lecube *et al.*, 2011). Nevertheless, many authors have reported alterations in Treg cells in terms of function in normoglycaemic autoantibody-positive individuals (Glisic-Milosavljevic *et al.*, 2007), or with risk-associated polymorphisms in high-risk haplotype but no evidence of disease (Garg *et al.*, 2012); as for other immune cell types, the question remains unanswered. Furthermore, hyperglycaemia could affect epigenetic mechanisms such as DNA methylation, histone modification and miRNA production, thus also contributing to dysfunctions in the immune system (Jerram *et al.*, 2017). Therefore, it would seem that it is the interplay between intrinsic defects and suboptimal metabolic control that drive the progression of the disease.

2.4.2. The role of the β-cell

Experts have alluded that β-cells are active contributors to T1D development and not only the innocent recipients of an unfortunate autoimmune attack (Soleimanpour and Stoffers, 2013). Indeed, several pieces of evidence point to underlying defects

in β-cells of patients with T1D which could partially contribute to or drive the autoimmune response in the disease. For instance, more than 50% of the **genes** associated with T1D are expressed in pancreatic β-cells, and they regulate their function and growth, signalling pathways and cytokine responses. Also, the fact that a relatively mild insulitis can lead a subject to require exogenous insulin administration together with the fact that β-cells reportedly survive for decades in the pancreas of these subjects (Keenan *et al.*, 2010) pushes scientists to think that **functional defects** in these β-cells are also involved in the pathogenesis of the disease.

For instance, β-cells are extremely sensitive to abrupt changes in glycaemia, and chronic exposure to high glycaemia levels has been reported as detrimental to β-cell insulin secretion and thriving (Cernea and Dobreanu, 2013). This **glucotoxicity** can damage β-cell function and survival through different mechanisms, which include loss of insulin gene expression, endoplasmic reticulum (ER) stress and unfolded protein response due to high demand of insulin secretion, excessive generation of reactive oxygen species and oxidative stress, impaired mitochondrial function and activation of NF-κB and Fas receptor signalling pathways that could trigger apoptosis. Federici *et al.*, 2001 provided evidence that human islets cultured in a high glucose medium upregulate the expression of pro-apoptotic genes. Thus, glucotoxicity can lead to insulin loss and hyperglycaemia, which in turn would aggravate glucotoxicity, further deteriorating β-cell function in a vicious cycle.

From an **immunological** perspective, β-cells are also significant partakers in disease progression. In autopsy studies of patients with T1D, β-cells exhibited the expression of abnormally high levels of HLA class I molecules and IFN-α, which could be necessary to recruit autoreactive T lymphocytes and engage cytotoxic $CD8^+$ T lymphocytes (Foulis *et al.*, 1987). Additionally, β-cells can upregulate the expression of adhesion and HLA class I molecules (Somoza *et al.*, 1994), and express HLA class II ectopically in response to inflammation (Pujol-Borrell *et al.*, 1986; Vives-Pi *et al.*, 1996). Also, two recent reports have demonstrated that β-cells express high levels of PD-L1 in association with insulitis (Osum *et al.*, 2018) and caused by IFN-α and IFN-γ stimulation (Colli *et al.*, 2018), likely in the attempt to attenuate the autoimmune attack. Interestingly, when released from this pro-inflammatory environment, cultured β-cells from patients with T1D exhibit a normalised biphasic insulin secretion (Krogvold *et al.*, 2015), demonstrating that this environment and its effects on β-cells are crucial in T1D pathogenesis.

Physiological massive peaks of β-cell apoptosis could also contribute to T1D development. These peaks have been observed during prenatal life (Meier *et al.*, 2010) and after birth (Kassem *et al.*, 2000), coinciding with endocrine pancreas remodelling stages. As aforementioned (see section 1.1.3), when a significant number of cells undergo apoptosis or the clearance capacity of phagocytes is not able

to meet the demand, apoptotic cells can potentially turn into secondary necrotic cells. Their released danger signals could exacerbate the pro-inflammatory milieu and contribute to the immunogenic presentation of autoantigens, a fact that has been proposed as one of the first events in autoimmune diseases (Wickman *et al.*, 2012). As mentioned in section 2.4.1, macrophages from patients with T1D have shown defects in apoptotic cell phagocytosis (Geerlings and Hoepelman, 1999), further suggesting that deficient efferocytosis may be involved in the pathogenesis of the disease.

Moreover, β-cells are particularly sensitive to induced **apoptosis**. The leukocytic infiltrate releases cytokines, such as IL-1β, TNF-α and IFN-γ, and reactive oxygen species that can act coordinately to promote β-cell apoptosis (Rojas *et al.*, 2018; Soleimanpour and Stoffers, 2013). Likewise, the very same β-cells are known to produce IL-1β (Reddy *et al.*, 2018), and Fas and Fas ligand expression have been reported in inflamed islets (Moriwaki *et al.*, 1999). This pro-inflammatory microenvironment also activates the NF-κB signalling pathway which, aside from inducing the expression of pro-apoptotic genes, regulates anti-apoptotic genes. One of such is the *TNFAIP3*, a gene that has been described as a genetic risk factor in T1D development (Barrett *et al.*, 2009). As previously stated (see section 2.4.1), its function is to inhibit the NF-κB signalling pathway in APCs but, in β-cells, it inhibits the production and signalling of the nitric oxide pathway, mediating a cytoprotective effect and preventing apoptosis (Grey *et al.*, 1999; Størling and Pociot, 2017).

Another mechanism theorised as capable of fuelling the autoimmune response in T1D could be the slow and small, but present nonetheless, **regeneration** capacity of the β-cells. Indeed, residual insulin production and β-cell turnover still take place, albeit residually, in patients with more than 50 years of disease evolution (Keenan *et al.*, 2010). Three putative mechanisms could contribute to increasing β-cell mass, these being the replication of existing β-cells, neogenesis of β-cells from precursor cells residing in the pancreatic ducts and transdifferentiation from other endocrine lineage cells in the islets, such as α-cells and δ-cells (Baeyens *et al.*, 2018). Nevertheless, the recovery of β-cell mass stands as a double-edged sword in the disease pathogenesis: on the one hand, β-cell regeneration could try to compensate for the β-cell mass lost by the autoimmune attack but, on the other hand, it constitutes a new source of autoantigens to feed the same autoimmune response. Interestingly, proteins from the REG family, which contribute to the regeneration of β-cells, are overexpressed in inflamed conditions (Gurr *et al.*, 2002) and act as autoantigens in experimental autoimmune models of T1D (Gurr *et al.*, 2007; Planas *et al.*, 2006).

Finally, **maternal microchimerism**, which refers to the significant presence of cells from a maternal origin in autoimmune lesions, has drawn attention in the last years. The placental exchange allows for these cells to circulate into the foetus and,

although being semi-allogenic, they can survive untouched by the immune system of the host during their whole life. Moreover, they have been found in peripheral blood (Nelson *et al.*, 2007; vanZyl *et al.*, 2010) and pancreata of patients with T1D and, interestingly, they have a pancreatic phenotype and express endocrine, exocrine and ductal antigens (Ye *et al.*, 2014a). Since these cells do express insulin, they have been theorised as capable of releasing autoantigens and feeding the autoimmune response. Currently, the function of these cells in the adult, the mechanisms involved in their tolerance or their role in T1D pathogenesis is not understood (Ye *et al.*, 2014b).

2.4.3. Rodent experimental models in insulin-dependent diabetes

To study T1D, both spontaneous and induced experimental models are available. Concerning the former, the **non-obese diabetic (NOD) mouse** has raised as the gold-standard, most widely used mouse model of T1D, as it suffers a type of spontaneous autoimmune diabetes that is very similar to the human disease (Côrte-Real *et al.*, 2009). This inbred strain was established in 1980 by Makino and colleagues by selecting cataract-prone mice strains (Makino *et al.*, 1980). The autoimmune process in NOD mice sets off at 3-4 weeks of age, with a leukocytic infiltration that surrounds the pancreatic islets (peri-insulitis) and progresses steadily to the core. At the prediabetic stage, pancreatic islets are primarily infiltrated with $CD4^+$ and $CD8^+$ T lymphocytes, although NK cells, macrophages, DCs and B cells can also be found (King, 2012). In the end, they collaborate to eliminate pancreatic β-cells, but $CD8^+$ T lymphocytes are the main effectors of β-cell killing by direct cytotoxicity (Pirot *et al.*, 2008). On the subject of insulitis, however, two noteworthy aspects are the entirely different islet cytoarchitecture and the histopathology of insulitic lesions between NOD mice and humans. First, while mice exhibit non-β cells encircling β-cells located at the centre of the islets, endocrine cells in human islets are distributed throughout the islets without distinct central and peripheral areas (In't Veld, 2014). Moreover, the NOD mouse shows massive lymphocytic infiltration in the islets affected by insulitis, whereas the human disease is characterised by a relatively mild islet infiltration and a significant number of unaffected islets (Morgan and Richardson, 2018). Nevertheless, when these mice become overtly diabetic, glucosuria is present alongside the typical clinical signs of diabetes, and they require insulin treatment to survive (Gvazava *et al.*, 2018).

As is the case in humans, development of T1D in NOD mice bears a polygenic base of susceptibility, and the specific loci have been named as insulin-dependent diabetes (Idd). The primary genetic contributors to T1D predisposition are the MHC class II genes and, among them, the MHC class II allele $I\text{-}A^{g7}$, which is the homolog gene to the DQ8 allele in humans (Wen *et al.*, 2002). Other genes located outside

the MHC locus also confer susceptibility, such as the *Ctla4*, the *Il2* and the *Ptpn8* genes, the mouse ortholog of human *PTPN22* gene (Wicker *et al.*, 2005). Moreover, as in humans, autoantibodies to insulin can be found in NOD mice sera, even though several other common target autoantigens have been found —proinsulin, insulin, GAD and IA-2, among others (Bonifacio *et al.*, 2001; Zhang *et al.*, 2008). It is worth noting that, unlike humans, mice have two insulin genes, *Insulin gene 1* and *Insulin gene 2*, the first being a rodent-specific retrogene and the second being an ortholog of the insulin gene found in other mammals (Shiao *et al.*, 2008). Interestingly, both genes are expressed in β-cells, although *Insulin gene 2* expression predominates in the thymus and ocular tissues of the mice. Additionally, the autoimmune process in NOD mice has also been shown to be influenced by environmental factors. Development of the disease in these mice is positively associated with small microbial exposure, meaning that mice must be kept in specific-pathogen free (SPF) conditions to achieve a high incidence of diabetes (Alam *et al.*, 2011), curiously in accordance with the *Hygiene hypothesis* described in section 2.3.2. Indeed, these parallels between NOD mice and humans have been useful in dissecting T1D pathophysiology, and especially for studying the prediabetic stage and test various procedures to stop disease progression. However, contrary to human disease, female NOD mice present an earlier onset —12 weeks of age— and a higher incidence of T1D than their male counterparts, this being 80-90% and 20-30% at 30 weeks of age, respectively (King, 2012). Thus, sex hormones are believed to modulate disease progression in mice, and testosterone has been demonstrated to exert protective effects in this experimental model since castrated males develop diabetes with the same incidence as intact females (Makino *et al.*, 1981).

In addition to autoimmune diabetes, NOD mice are prone to other autoimmune diseases such as thyroiditis, sialadenitis, peripheral polyneuropathy, prostatitis and haemolytic anaemia (Bour-Jordan *et al.*, 2013). Development of these syndromes could come as a result of the several dysfunctions that the immune system of NOD mice harbours, including defects in maturation and function of APCs and low numbers of NK, iNKT cells and $CD4^+CD25^+$ Treg cells (Anderson and Bluestone, 2005). In this sense, many differences exist between the murine and the human immune system (Mestas and Hughes, 2014) and between murine and human T1D (Reed and Herold, 2015). Perhaps it is due to these differences that numerous drugs have failed to translate their effectiveness in the animal model into humans (von Herrath and Nepom, 2009).

To further dissect relevant aspects of the disease *in vivo*, countless genetically-modified variants of NOD mice have been generated in the last few years. For instance, the generation of an immunodeficient variant (NOD-*scid IL2rg*^{null}) has allowed for the transplantation of human PBMCs and engraftment of several tissues,

and humanised mice —NOD mice harbouring human alleles— have permitted the testing of encouraging therapies preclinically before their translation into clinical trials (Pearson *et al.*, 2016).

The **BioBreeding (BB)** rat is another spontaneous model for autoimmune diabetes (Nakhooda *et al.*, 1977), in which the disease develops between 8 and 16 weeks of age and affects both genders similarly (Gvazava *et al.*, 2018). This model presents insulitis composed of T cells, B cells, macrophages and NK cells, but has severe lymphopenia of $CD4^+$ T cells and complete absence of $CD8^+$ T cells, which poses as a considerable drawback. Nevertheless, BB rats have been used in intervention studies, induction of tolerance after islet transplantation and diabetic neuropathy assays (King, 2012). On the other hand, the **LEW.1AR1/-iddm rats**, which bear the defined LEW.1AR1 MHC haplotype, develop diabetes within the week after insulitis has set off at 8-9 weeks of age (Jörns *et al.*, 2005; Lenzen *et al.*, 2001). Since it survives well after the onset of diabetes, it has been mainly used to study mechanisms involved in diabetic complications (King, 2012).

As for induced models, they can be chemically-induced, genetically-induced or virally-induced, and they are useful to study diabetes without the interference of the immune system. **Streptozotocin** (STZ), synthesised by *Streptomyces achromogenes*, is the predominant chemical compound used to induce diabetes. STZ enters the pancreatic β-cell through the Glut-2, causing alkylation of the DNA, reduction in cellular ATP, inhibition of insulin production and β-cell death (King, 2012). Moreover, the dosage of STZ determines the existence of two models: a single high dose (100-200 mg/kg in mice) leads to rapid ablation of the β-cells and hyperglycaemia in 48 to 72 hours, whereas multiple low doses (20-40 mg/kg/day in mice) induce insulitis and a reduction in insulin secretion, eventually leading to hyperglycaemia (Deeds *et al.*, 2011). On the other hand, the **AKITA mouse** is a genetically-induced model bearing a spontaneous mutation of the *Insulin 2* gene that prevents the correct processing of proinsulin, which causes ER stress and overt hyperglycaemia at 3-4 weeks of age (King, 2012). Hence, this model has mostly been used to study potential ER stress-ameliorating agents in the islets. Finally, specific **virus** have been used to induce diabetes in experimental models, such as the Coxsackie B virus, the encephalomyocarditis virus and the Kilham rat virus (King, 2012). Interestingly, the overexpression of the antiviral cytokine IFN-β in the islets is capable of accelerating the disease in NOD mice, as demonstrated in the transgenic model NOD RIP-IFN-β that expresses human IFN-β under the control of the insulin promoter (Alba *et al.*, 2004). This model develops autoimmune diabetes at 3-4 weeks of age with similar incidence in males and females and resembling the disease presented by very young children, thus pointing to the significant influence that microbial agents could have in the pathogenesis of the disease.

Thus, albeit imperfect, experimental models remain a solid foundation in the study of the natural history of T1D and in testing promising preventive and therapeutic strategies. It is essential, however, to be aware of the advantages and drawbacks and the discrepancies existent between each model and the human disease, so that preclinical research and therapeutic approaches can be successfully translated into the clinics.

3. Multiple sclerosis

MS is an immune-mediated disorder that affects the brain and spinal cord of the central nervous system (CNS), caused by a misguided autoimmune response against myelin-associated antigens produced by oligodendrocytes. This injury results in the deterioration of the myelin sheaths covering the axons of the nerve cells, hindering the proper conduction of electrical transmission. Although the course is highly variable among individuals, MS ultimately leads to severe physical and cognitive impairments in most patients. That is why MS stands as a significant cause of neurological disability in young adults.

3.1. Natural history of the disease

The nervous system controls and integrates functional activities of the organs, adapting continually to changes in the inner and outer environments. The nerve tissue of the CNS, which comprises the brain and spinal cord, is made of neurons and neuroglial or supporting cells. The neuroglial cells of the CNS provide metabolic and mechanical support and protection to the neurons, and they include astrocytes, microglial cells and oligodendrocytes, among others. Astrocytes take up ions and neurotransmitters released into the extracellular space, whereas microglial cells function as phagocytes in clearing debris and damaged structures. On the other hand, oligodendrocytes provide electrical insulation to CNS neurons, since concentric layers of their plasma membrane form the myelin sheaths that cover the axons. Based on this, the CNS can be further classified into white matter, which contains myelinated axons of nerve cells and associated neuroglial cells, and grey matter, which consists of an aggregation of neuronal cell bodies, unmyelinated portions of axons and neuroglial cells (Gartner and Hiatt, 2014). Importantly, astrocytes located at the periphery of the CNS form a continuous layer over the blood vessels and maintain the blood-brain barrier (BBB), which is an endothelial barrier that prevents the free passage of particular blood-borne substances into the nerve tissue.

Focusing on MS, the autoimmune attack is directed against several antigens of the myelin made from oligodendrocytes in the CNS. It is worth mentioning that myelin from the peripheral nervous system is produced by Schwann cells and expresses

different myelin-specific proteins, perhaps explaining why this myelin escapes the autoimmune attack (Gartner and Hiatt, 2014). Despite the fact that many efforts have been made, the trigger mechanisms of MS remain unknown and, as it happens in other autoimmune diseases, experts agree that MS is the result of several genetic, environmental and stochastic risk factors (Baecher-Allan *et al.*, 2018). The evidence suggests that it is the coordinated action of autoreactive $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and macrophages that causes the inflammatory episodes characteristic of MS, provided that the BBB allows free passage of these cells (Goodin, 2014). These cells cause injury to the myelin sheaths, to the oligodendrocytes and, to a lesser extent, to nerve cells, nerve processes and axons. Thus, the main hallmarks of this disease are demyelination, axonal loss and gliosis, which concentrate on the white matter but often also affect the grey matter (Bo *et al.*, 2006). These attacks —named *flares*, *exacerbations* or *relapses*— recur at unpredictable intervals. Upon the occurrence of a flare, its symptoms and signs depend upon the lesion site in the CNS. The typical symptoms of these relapses include visual impairment (monocular visual loss, blurred vision and double vision), facial sensory loss, asymmetric limb weakness, asthenia, spasticity, cerebellar ataxia, sphincter dysfunction, Lhermitte's syndrome and cognitive impairment, among others (Brownlee *et al.*, 2017). These are due to optic neuritis, transverse myelitis, brain-stem dysfunction and cerebellar lesions (Reich *et al.*, 2018).

Early and accurate diagnosis of MS is crucial for patients since clinical trials with different immunomodulatory therapies have demonstrated that their early introduction is superior in terms of delaying the next attack, reducing the number of new lesions on the CNS, slowing disability progression and cognitive impairment (Hurwitz, 2009). The diagnosis of MS is based on neurological signs and symptoms and evidence of MS lesions in the CNS observed by magnetic resonance imaging (MRI) scanning. This technique is able to highlight three different types of findings, which appear in accordance with the pulse sequence used (Kaunzner and Gauthier, 2017). Specifically, dark areas (hypointensities) in T1-weighted MRI scans reveal permanent myelin loss and axonal damage and loss, whereas T2-weighted images show total lesion load (disease burden). Moreover, gadolinium-enhanced T1-weighted MRI scans highlight the presence of new or enlarging active inflammatory lesions, since gadolinium is only able to enter the CNS through the disrupted BBB sites. Lesions suggestive of MS are present in 80% MRI scans of patients having endured the first acute attack and in most patients with established MS (Fisniku *et al.*, 2008), and even spinal cord lesions occur in 50% of patients undergoing the first exacerbation and 80-90% of patients with established disease (Bot *et al.*, 2002; Sombekke *et al.*, 2013). In order to make the diagnosis of MS, however, physicians must find evidence of two CNS lesions compatible with MS, which must appear disseminated in

space and time, and rule out any other probable causes for those lesions (McDonald *et al.*, 2001; Thompson *et al.*, 2018). Also, MRI scanning is used in the clinical monitoring of patients and in the testing of the beneficial effect of new therapies.

MRI is often sufficient to confirm the diagnosis of MS when the abnormal MRI scan is accompanied by the clinical syndrome, but the examination of the cerebrospinal fluid (CSF) can provide supporting evidence. The findings in this fluid include a mildly raised cell count (>5 cells per cm^3, mainly lymphocytes), an increased IgG index (>0.7) and presence of oligoclonal IgG bands (>2) that are produced intrathecally and thus are absent from the serum (Luque and Jaffe, 2007; Pröbstel *et al.*, 2015). In patients with MS, it is not uncommon to find non-specific autoantibodies, such as antinuclear antibodies, although they may not be clinically relevant (Negrotto *et al.*, 2015). Additional information can be provided by evoked potentials, which measure the electrical activity of the brain to detect the slowing of electrical conduction of specific visual, sensory, and auditory pathways due to demyelination, and neurological testing (Brownlee *et al.*, 2017). Throughout the progression of the disease, the 10-point Expanded Disability Status Scale (EDSS) allows for the assessment of the progressing disability in patients with MS over time (Kurtzke, 1983).

In general terms, five different clinical courses are recognised in MS disease (**Figure 4**, Brownlee *et al.*, 2017; Fisniku *et al.*, 2008; Goodin, 2014; Miller *et al.*, 2012):

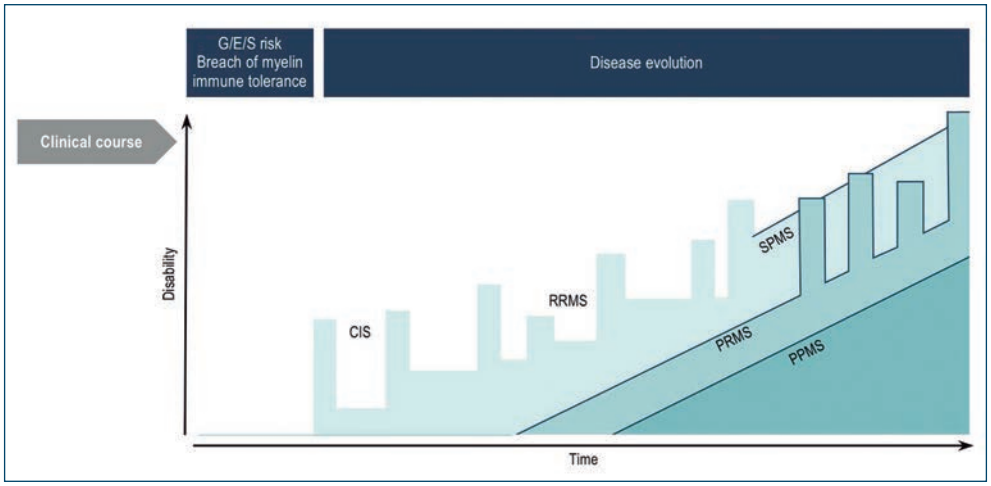


Figure 4. ***Stages of MS.*** *In individuals with genetic factors of susceptibility for MS, exposure to specific environmental factors and occurrence of specific stochastic factors (G/E/S risk) could lead to the development of an autoimmune response against myelin antigens. The CIS period marks the first neurological relapse consistent with an MS acute attack, and can eventually evolve into RRMS, in which the bars denote events causing inflammatory damage to the CNS. RRMS most frequently leads to SPMS, when there is a steadily progressive neurological disability. PPMS courses similarly to SPMS, without distinct relapses, whereas patients with PRMS exhibit acute attacks. Adapted from Baecher-Allan* et al.*, 2018 and Olsson* et al.*, 2017; infographic by VRB and SRF.*

- **Clinically isolated syndrome (CIS)**. It is recognised as the first episode of neurological dysfunction that is consistent with an MS relapse, and it lasts for 24 hours at minimum and occurs in the absence of fever or infection. By definition, a CIS is isolated in time (monophasic) and space (monofocal), but MS can be diagnosed during CIS if MRI findings confirm that an earlier inflammatory demyelinating episode occurred in a different location in the CNS —demonstrating dissemination in time and space—, even if it did not produce any clinical signs. After this event, the patient may or may not go on to develop MS, although more than 80% of patients with CIS do develop clinically definite MS within the next 20 years.
- **Relapsing-remitting MS (RRMS)**. This form is characterised by recurrent relapses, which vary in frequency and severity. These relapses develop acutely, and the deficits persist for a minimum of 24 hours. The final phase is the period of remission, and patients remain clinically stable until the next attack. However, the recovery of neurologic function and improvement during the remission phase is variable, and so residual disability could be accumulated over consequent relapses. On MRI scanning, patients show evidence of both acute and chronic inflammation. RRMS is the most prevalent form of MS, affecting 85-90% of patients. It typically affects young adults —on average being 20-30 year old—, and displays a notable gender bias, with a higher incidence among women (2-3:1).
  - Some patients experience a subset of RRMS that is very mild in nature —often referred to as **benign MS**— in which they are barely affected or accumulate little disability after 15 years of evolution. Since there are no biomarkers of this disease form and the course of MS and relapses cannot be predicted, this diagnosis can only be made in retrospect.
- **Secondary progressive MS (SPMS)**. It begins as RRMS does but, at some point, the disease course is altered so that exacerbations become less frequent and the patient begins to experience a steady deterioration in neurologic function, which is independent of any acute relapses that may or may not occur. In fact, 90% of patients with RRMS evolve into the SPMS form after decades with the disease. On MRI scanning, evidence of acute inflammation is discreet, whereas brain atrophy becomes more prominent. This form accounts for most of the neurologic disability reported with this illness.
- **Primary progressive MS (PPMS)**. The onset of the disease is insidious and, from the first moment, the patient experiences a steady decline in neurologic function without any distinct relapses. These patients show less evidence of active inflammation on MRI scans when compared to patients with RRMS. It affects only 10% of patients with MS, with similar frequencies in men and women, and has a later age of onset (between 40 and 50 years of age). Also,

this form presents a worse prognosis for ultimate disability compared to patients with RRMS, with over 50% of cases requiring ambulatory assistance within the first decade of disease.

- **Progressive-relapsing MS (PRMS)**. It starts with a progressive course very similar to that of PPMS, but the patients begin to experience occasional clinical attacks at some point, which are superimposed on the steadily progressive disease course. Only 5% of patients with MS experience this disease course.

In the last decade, however, the clinical classification of MS has been revised. Experts now agree that these clinical courses can be further classified into *active* or *not-active* clinical courses by considering the clinical progression of the patient, the presence of inflammatory processes from imaging techniques and the input from other paraclinical studies (Lublin *et al.*, 2014). Also, the considerable therapeutic effect of the currently available treatments has led to the introduction of the concept *no evidence of disease activity* (NEDA), which refers to the complete remission of the disease without ruling out any recurrences. The NEDA state is confirmed by the absence of relapses, new or enlarged MRI lesions and neurological disability worsening (Giovannoni *et al.*, 2018).

3.1.1. Available treatments

Currently, the available armamentarium for MS only include agents that modulate or suppress general pathways of the immune response (**Table 2**). Two types of treatment exist for MS: the ones that are given to treat acute relapses or exacerbations, and disease-modifying drugs, which alter the course of the disease in terms of exacerbation and lesion reduction, disease activity limitation and overall delaying its progression (Hamidi *et al.*, 2018; Torkildsen *et al.*, 2016). At the moment, the availability of therapies capable of preventing or reversing the progressive neurological deterioration is an unmet need. As a result of these immunomodulatory treatments, undesired immunosuppression and downmodulation of the immune response are frequent secondary effects in patients with MS. These include lymphopenia, increased risk of malignancies, flu-like symptoms in the case of IFN-β treatment and viral reactivation in the case of dimethyl fumarate and natalizumab treatments (Baecher-Allan *et al.*, 2018). Particularly, JC polyomavirus reactivation causes the lytic infection of glial cells, a condition known as progressive multifocal leukoencephalopathy (PML) and that is potentially fatal in patients treated with these immunosuppressive agents. Other common secondary effects include liver toxicity and skin reactions. Furthermore, a proportion of patients continue to experience clinical and MRI disease activity despite the treatment, thus categorised as *non-responders*. Although this can occur spontaneous-

Table 2. List of the main treatments approved for MS

Agent	Administration route	Disease indication	Therapeutic effect
TREATMENT OF ACUTE RELAPSE OR EXACERBATION			
Corticosteroid (prednisolone and methylprednisolone)	Intravenous	RRMS	Steroid hormone. Induces general immunosuppression and accelerates the recovery from relapses and limits neurological deficits.
DISEASE-MODIFYING DRUGS			
Alemtuzumab	Intravenous	RRMS	Anti-CD52 humanised mAb. Depletes B and T cells via ADCC and complement.
Cladribine	Oral	RRMS	Adenosine deaminase. Induces lymphocyte apoptosis and reduces $CD4^+$ and $CD8^+$ T cells and B cells.
Daclizumab	Intravenous	RRMS	Anti-CD25 humanised mAb. Halts IL-2 signalling pathway and increases $CD56^+$ NK activity.
Dimethyl fumarate	Oral	RRMS	Chemical agent. Reduces memory T cells and shifts to anti-inflammatory Th2 state. Could have a neuroprotective effect. Mechanism of action not completely understood.
Fingolimod	Oral	RRMS	S1PR agonist. Inhibits lymphocytic egress and traps lymphocytes within lymph nodes, inhibits DCs migration to secondary lymphoid organs.
Glatiramer acetate	Subcutaneous	RRMS	Random amino acid polymers to bind to MHC in order to compete with the binding peptide. Increases anti-inflammatory cytokine production, $CD8^+$ Treg cells and type II monocytes.
IFN-β (1a and 1b) and pegylated IFN-β	Intramuscular or subcutaneous	RRMS	Ligand to type I IFN receptor. Inhibits T cell division, BBB extravasation and pro-inflammatory cytokine production, and increases Treg cell induction.
Ocrelizumab	Intravenous	RRMS, PPMS	Anti-CD20 humanised mAb. Depletes $CD20^+$ B cells and pathogenic B cell antigen presentation.
Mitoxantrone	Intravenous	RRMS, SPMS	Anti-neoplastic agent. Interferes with DNA repair and causes DNA strand breaks. Inhibits lymphocyte and monocyte migration, B cell function and secretion of pro-inflammatory cytokines.
Natalizumab	Intravenous	RRMS	Anti-CD49d (α4 subunit of VLA-4 integrin) humanised mAb. Blocks VLA-4 binding to VCAM-1, and thus inhibits T cell and B cell migration into the CNS.
Teriflunomide	Oral	RRMS	Dihydroorotate dehydrogenase blocker. Inhibits pyrimidine synthesis, T cell activation and pro-inflammatory cytokine secretion.

RRMS: relapsing-remitting multiple sclerosis; mAb: monoclonal antibody; ADCC: antibody-dependent cellular cytotoxicity; NK: natural killer; S1PR: sphingosine-1-phosphate receptor; DCs: dendritic cells; MHC: major histocompatibility complex; IFN: interferon; BBB: blood-brain barrier; PPMS: primary progressive multiple sclerosis; SPMS: secondary progressive multiple sclerosis; CNS: central nervous system. Adapted from Baecher-Allan *et al.*, 2018 and Torkildsen *et al.*, 2016.

ly, a significant part of patients does become *non-responder* after developing neutralising antibodies against these biopharmaceuticals (Lundkvist Ryner *et al.*, 2014).

3.2. Epidemiology

MS is one of the most common diseases coursing with disability in young adults (Hurwitz, 2009). Its incidence in Europe is estimated at 4.3 cases per 100,000 inhabitants per year (Pugliatti *et al.*, 2006), and is reportedly increasing in the last years (Benito-León and Bermejo-Pareja, 2010; Grytten *et al.*, 2016). As with T1D, the distribution of MS displays a marked North-South gradient, and even the age of onset is earlier in countries at higher latitudes (Tao *et al.*, 2016). Europe and North America display the highest prevalence rates (>100 cases in 100,000 inhabitants), whereas the prevalence rate in Eastern Asia and sub-Saharan Africa is significantly lower (2 cases in 100,000 inhabitants) (Leray *et al.*, 2016). In Spain, its prevalence ranges between 80 and 100 cases per 100,000 inhabitants, affecting 46,000 people (Multiple Sclerosis International Federation, 2013). It is estimated that MS affects more than 2.3 million people worldwide (Feigin *et al.*, 2017).

Patients with MS are usually diagnosed in their 20s or 30s, although cases in young children and older adults have been described (Goodin, 2014). Furthermore, MS is at least two to three times commoner in women than in men (Alonso and Hernán, 2008), as is the general rule for most autoimmune diseases.

3.3. Aetiology

As was the case in T1D, MS is recognised as a multifactorial disease with a complex aetiologic base. Monozygotic twins display a concordance rate of 20-30% in northern populations (Islam *et al.*, 2006), suggesting that additional environmental or epigenetic factors influence the development of MS in genetically-predisposed individuals.

3.3.1. Genetic factors

MS has a multifactorial genetic component. The best-established association is mapped to the HLA region, and especially to the allele DR15 (HLA-DRB1*1501) in both northern European and North American populations (Goodin, 2014). In fact, heterozygotes for this allele have an odds ratio of >3, whereas in homozygotes, this increases to >6 (Hollenbach and Oksenberg, 2015). Other alleles associated with MS include DQB2*0602, DRB1*1303, DRB1*0301, DRB1*0801, DQB1*0302, and DRB1*0405-DQA1*0301-DQB1*0302 in Mediterranean populations (Compston and Coles, 2002). Specific class I alleles, such as HLA-A*0201 and HLA-B*4402,

HLA-B*3801 and HLA-B*5501, could have protective effects (Didonna and Oksenberg, 2017; Moutsianas *et al.*, 2015).

Subsequent GWAS have found more than 100 genetic variants associated with the disease, with many of them reportedly involved in immune response and associated with other autoimmune diseases (Beecham *et al.*, 2013). Among them, the *IL2RA*, *IL7RA*, *TNFR1*, *TNFRSF1A*, *IRF8*, *CD6*, *CD25*, *CD58*, *CD226, BAFF, TYK2,* and *CYP2R1* genes and other genes involved in vitamin D metabolism have gained special attention (Dobson and Giovannoni, 2019; Sawcer *et al.*, 2011; Sospedra and Martin, 2016).

Also, several studies have identified that differences exist in epigenetic modifications when comparing patients with MS and control subjects (Olsson *et al.*, 2017).

3.3.2. Environmental factors

Apart from predisposing genetic factors, diverse environmental phenomena have shown association with MS development. For instance, a high number of studies demonstrate that the **migration** from one geographic area to another can alter a subject's risk of developing MS peculiarly (Ahlgren *et al.*, 2012; Cabre *et al.*, 2005; Dean and Kurtzke, 1971). When individuals migrate prior to their adolescent years, their MS risk increases or decreases to meet the risk of the host region, and even children born from immigrants show an MS risk similar to that of their birth country (Elian *et al.*, 1990).

Microorganism infections have also attracted considerable interest as potential triggers for MS, with Epstein-Barr virus (EBV) emerging as the most prominent. EBV belongs to the herpes family of virus and targets specifically the epithelial cells of the oropharynx and B cells. This infection typically occurs during early childhood and currently affects 90% of the population (Nielsen *et al.*, 2007). If the EBV initial infection is delayed until adolescence or later, or if the subject develops symptomatic mononucleosis, the risk of MS increases significantly (Thacker *et al.*, 2006), and essentially all patients with adult-onset MS have evidence of prior EBV infection (Goodin, 2014). Interestingly, Serafini *et al.*, 2007 found evidence of EBV infection in a significant percentage of B cells infiltrating the CNS in post-mortem samples of patients with MS. Among other evidence, patients with MS have shown elevated titres of anti-EBV antibodies in the serum and increased EBV-specific $CD8^+$ T cell frequencies during active MS (Angelini *et al.*, 2013; Pender and Burrows, 2014). The mechanisms proposed to explain this association are the activation of innate immunity by EBV-encoded small RNA molecules in the CNS, cross-reactivity between EBV and CNS antigens, expression of αB-crystallin in EBV-infected B cells that elicits a $CD4^+$ T cell response against such protein expressed in oligodendrocytes, bystander damage to the CNS by EBV-

specific $CD8^+$ T cells and EBV infection of autoreactive B cells (Pender and Burrows, 2014). That is why experts argue that EBV infection is necessary —but not sufficient— for a subject to develop MS. As for the involvement of other infectious agents in MS pathogenesis, such as human herpesvirus-6 and *Chlamydia pneumoniae*, the evidence is not quite as compelling (Goodin, 2014). By contrast, viral infections have been associated with MS relapses (Correale *et al.*, 2006; Sibley *et al.*, 1985), as evidenced by exacerbations rates being increased 3-fold after infection, suggesting that **molecular mimicry or cross-recognition** could be involved in the perpetuation of MS.

The existence of a latitudinal gradient in MS indicates that **ultraviolet radiation** and probably **vitamin D levels** can influence the risk of developing MS (Simpson *et al.*, 2011). Ultraviolet radiation and increased vitamin D levels, especially before young adulthood, are associated with protection from MS (Lucas *et al.*, 2015; Munger *et al.*, 2006). Furthermore, after vitamin D binds to its receptor in the cell surface, the complex is internalised and translocated to the nucleus, where it binds to the vitamin D response element located in many genes (Goodin, 2014). Strikingly, one of these response elements is situated in the promoter region of the HLA-DRB1*1501 allele (Ramagopalan *et al.*, 2009), which is recognised as one of the most critical alleles involved in MS aetiology. Also, a **month-of-birth effect** has also been raised as predisposing for MS. Several studies from Canada and Northern Europe have found that more patients with MS were born in May and fewer were born in November (Sadovnick *et al.*, 2007; Willer *et al.*, 2005), whereas in the southern hemisphere, these findings are entirely reversed (Staples *et al.*, 2010; Willer *et al.*, 2005). Perhaps the reason for these findings could be that, in the northern hemisphere, mothers of babies born in May spend much of their pregnancy during winter months —when there is less sun exposure— in comparison to mothers of babies born in November, who are pregnant during the summer (Goodin, 2014). During gestation, human placenta responds and synthesises vitamin D, and so it could regulate immune responses in this period (Evans *et al.*, 2006; Shin *et al.*, 2010). In close relation to the last finding, a **maternal effect** is also argued to confer risk of developing the disease. Firstly, half-siblings concordant with MS are more likely to share their mother than their father (Ebers *et al.*, 2004). Secondly, MS concordance rate in dizygotic twins is higher than that of non-twin siblings (Compston and Coles, 2002), with one Canadian study reporting a concordance rate of 2.9% in non-twin siblings and 5.4% in dizygotic twins, who share the intrauterine environment (Willer *et al.*, 2003). These two observations suggest that MS susceptibility could be transmitted from mother to child, although the mechanism is not understood.

Several studies have hinted at the possibility that the **microbiome** could have a role in disease pathogenesis. Gut microbial dysbiosis has been found in patients with MS when compared to control subjects (Chen *et al.*, 2016; Jangi *et al.*, 2016; Miyake *et al.*, 2015), and increased frequency of intestinal Th17 correlates with microbial

alteration and disease activity (Cosorich *et al.*, 2017). To date, however, no specific diet is associated with an increased risk of MS. Vitamin A and D, salt, omega-3-unsaturated fatty acids and polyphenol have been proposed with the ability to influence MS risk, and Farez *et al.*, 2015 reported that a higher sodium and salt intake was associated with increased risk of MS disease. By contrast, coffee and alcohol consumption are associated with decreased risk (Olsson *et al.*, 2017). Nonetheless, several studies exist associating early childhood and adolescent **obesity** with increased MS susceptibility, particularly in women (Gianfrancesco and Barcellos, 2016). Obesity is often related to dysbiosis in gut microbiota, is characterised by a low-grade inflammatory response and suboptimal immune system function and correlates with lower levels of vitamin D in the circulation. The possibility exists that it is the combination of all these factors that ultimately impacts on MS risk.

Tobacco smoking has also been linked to both MS risk and disease activity (Wingerchuk, 2012), perhaps through the driving of Th17 responses in the lung or alteration of vascular permeability in the CNS (Hedström *et al.*, 2011; Jafari and Hintzen, 2011). Several case-control studies have demonstrated a higher proportion of MS cases in smokers than in non-smokers (Goodin, 2014), and even passive exposure to tobacco smoke is associated with an early onset of MS in children (Hedström *et al.*, 2011; Mikaeloff *et al.*, 2007). Furthermore, smoking has been associated with the risk of developing neutralising antibodies against biologic treatments used for MS, such as natalizumab and IFN-β (Hedström *et al.*, 2014a, 2014b).

Finally, **lifestyle** and other related events have been associated with increased risk of MS, including physical trauma, psychologic stress, night shift work and toxic exposures (Goodin, 2014; Olsson *et al.*, 2017). Studies approaching these, however, reveal inconclusive data, perhaps because the underlying mechanisms for these factors to influence MS can be especially challenging to dissect. Interestingly, **vaccination** had been debated as a potential trigger for developing MS and as relapse inductor but, to date, accumulated evidence on influenza, varicella, rabies, pertussis, typhoid fever, cholera, hepatitis B, tetanus, meningitis, and Bacillus Calmette-Guérin vaccination does not prove any causal association (Mailand and Frederiksen, 2017).

Taken together, these data indicate that a combination, or even sequential occurrence, of these factors could be determinant in the ultimate development of MS in individuals genetically predisposed.

3.3.3. Stochastic factors

As it happens in T1D, the **random rearrangement of the TCR and BCR genes** and failure of specific **central and peripheral tolerance mechanisms** could significantly increase the risk of a subject to ultimately suffer from MS.

3.4. Pathological process in human MS

The MS pathological process is featured by the breakdown of the BBB, multifocal inflammation, demyelination, oligodendrocyte loss, reactive gliosis and axonal degeneration (Baecher-Allan *et al.*, 2018). The relapses in MS are driven by different immune cells that migrate into the CNS, an occurrence that can be modulated by several treatments that target different effector cells, prevent their trafficking into the CNS, or increase the number or function of regulatory cells. At some point, however, the disease enters the progression stage, a phase in which active inflammation fades and immune-independent mechanisms like mitochondrial injury, oxidative stress and ion imbalance gain importance. In fact, disease progression is directly associated with grey matter and cortical atrophy, a phenomenon that is probably exacerbated once the brain exhausts its capacity to compensate for further axonal loss (Nave and Trapp, 2008). To date, available therapeutics are unable to target the events inciting the progression of MS. Such a complex interplay of immune and pathological factors has been dissected employing tissues donated from deceased organ donors, clinical trials using several immunomodulatory treatments that describe the different affected pathways, and employing experimental models of disease, in a similar fashion that researchers in T1D have followed.

3.4.1. The role of the immune system

MS inflammatory lesions contain $CD4^+$ and $CD8^+$ T cells, B cells, plasma cells and macrophages, supporting the role of both the innate and the adaptive immune system as drivers of the disease (**Figure 5**, Steinman, 2014).

Concerning **$CD4^+$ T cells**, the *outside-in hypothesis* proposes that peripherally activated autoreactive effector $CD4^+$ T cells migrate into the CNS to initiate the disease. Afterwards, they would be activated locally by APCs and recruit additional T cells and macrophages to establish the inflammatory lesion (Baecher-Allan *et al.*, 2018). The homing of immune cells to the CNS is crucial for relapses to take place and involves a highly specific interaction between a particular integrin on the surface of lymphocytes —α4β1 or VLA-4— and a ligand on the surface of the brain endothelium —VCAM-1—, a discovery favoured by the beneficial effect of anti-VLA-4 natalizumab therapy in MS relapses (Steinman, 2012). In fact, high numbers of $CD4^+$ T cells accumulate in inflammatory lesions of the white matter at the centre of the lesion, and they are also found in the CSF of patients (Hauser *et al.*, 1986; Traugott *et al.*, 1983). Moreover, both Th1 and Th17 effector cells appear to have an active role in human disease, as demonstrated by increased production of either IFN-γ and IL-17 in MS lesions and clinical trials targeting these molecules (Elain *et al.*, 2014;

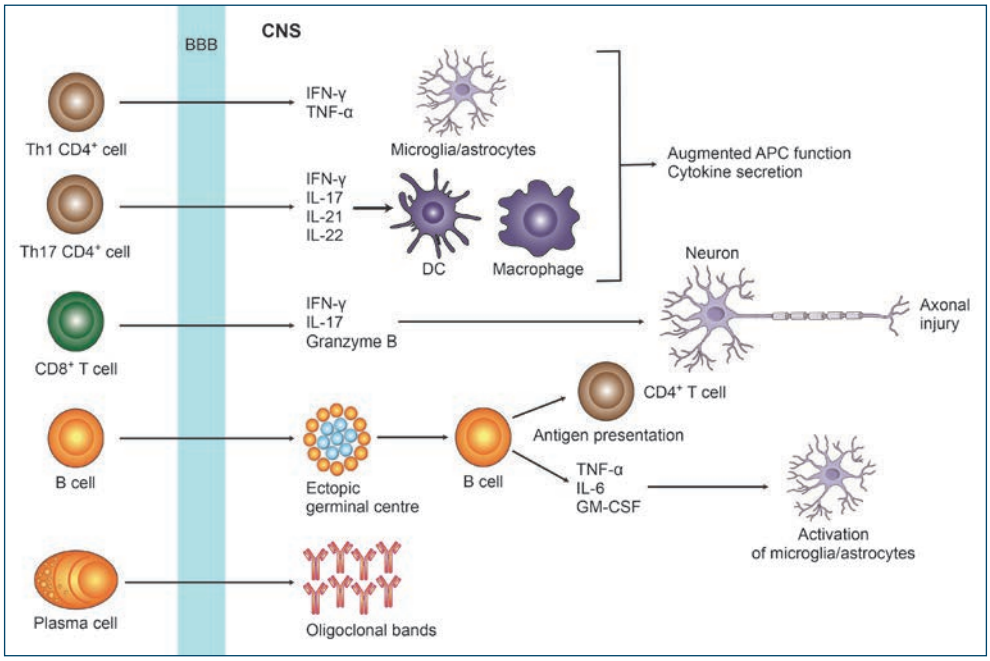


Figure 5. ***Main effectors of MS pathogenesis.*** *In this process, Th1 cells and Th17 cells are known to produce pro-inflammatory cytokines to activate the function and cytokine secretion of professional APCs (DCs and macrophages), microglia and astrocytes. Also, CD8⁺ T cells secrete pro-inflammatory cytokines and cytotoxic molecules that cause axonal injury. B cells are known to generate ectopic germinal centres within the CNS, act as APCs to CD4⁺ T cells and secrete cytokines to activate professional APCs and glial cells. Finally, plasma cells produce oligoclonal bands, which are biomarkers of this pathogenic immune response. Adapted from Baecher-Allan* et al.*, 2018; infographic by VRB and SRF.*

Havrdová *et al.*, 2016; Lock *et al.*, 2002; Panitch *et al.*, 1987). Interestingly, Th17 cells with the ability to secrete IFN-γ have been found in brains from patients with MS (Kebir *et al.*, 2009). Also, CCR6⁺ myelin-reactive T cells from patients with MS produce higher levels of IFN-γ, IL-17 and GM-CSF and lower levels of IL-10 than their counterparts obtained from control subjects (Cao *et al.*, 2015). To explain the role of both IFN-γ and IL-17 in the driving of the disease, experts argue that IFN-γ could increase the expression of HLA class I and HLA class II molecules and enhance the antigen-presenting capacity of macrophages, astrocytes and microglia (Gobin *et al.*, 2001; Keskinen *et al.*, 1997), whereas IL-17 would aid in the maintenance of the inflammatory response and the breakdown of the BBB by inducing reactive oxygen species production (Huppert *et al.*, 2010).

Whereas CD4⁺ T cells locate in the centre of lesions, **CD8⁺ T cells** are mostly found at the edges, also accumulating in active demyelinating plaques —where 50-fold CD8⁺ accumulate in relation to CD4⁺ T cells— and in cortical plaques

(Hauser *et al.*, 1986; Losy, 2013; Traugott *et al.*, 1983). Current findings point to a relevant role of $CD8^+$ T cells after the disease has been initiated by $CD4^+$ T cells. In fact, a portion of $CD8^+$ T cells isolated from the CNS of patients shows the same specificity and memory phenotype, pointing to the fact that they may have expanded *in situ* (Babbe *et al.*, 2000; Jacobsen *et al.*, 2002). Also, microglial cells can cross-present exogenous antigens in their MHC class I molecules, potentially activating myelin-reactive $CD8^+$ T cells (Zang *et al.*, 2004). Upon activation, $CD8^+$ T cells can secrete IFN-γ, IL-17 and granzyme B (Huber *et al.*, 2013), thus killing myelin-expressing cells and causing axonal damage (Melzer *et al.*, 2009). To support this, higher levels of granzyme have been found in the CSF of patients with active disease flares as compared to those in remission (Malmeström *et al.*, 2008), and $CD8^+$ T cell frequency correlates with axonal injury (Melzer *et al.*, 2009).

Because of the striking ameliorating effect that anti-CD20 therapy has demonstrated on MS, **B cells** are believed to be main partakers in the disease pathogenesis (Greenfield and Hauser, 2018). This treatment depletes B cells from the circulation and from the CSF in 5 months, reduces the appearance of gadolinium-enhanced lesions by an 88% and decreases significantly the number of T cells and the ability of the remaining ones to secrete IL-17 and IFN-γ (Baker *et al.*, 2017; Bourdette and Yadav, 2008; Naismith *et al.*, 2010). By contrast, it does not affect plasma cells nor their production of antibodies or oligoclonal bands. Because patients with MS possess high levels of memory B cells, short-lived plasmablasts and long-lived plasma cells in their CNS (Michel *et al.*, 2015) but only the former are targeted by anti-CD20 therapy, experts speculate that memory B cells have an instrumental pathogenic role in the disease. In this sense, B cells could serve as primary APCs to drive the activation of pathogenic T cells and as producers of chemokines and cytokines to enhance this response (Baecher-Allan *et al.*, 2018). In fact, B cells can cross the BBB and aid in the generation of ectopic germinal centres (Serafini *et al.*, 2004), which are identified in the meninges of patients with progressive MS, suggesting that B cells can undergo expansion and differentiation to plasma cells *in situ* (Magliozzi *et al.*, 2006). High amounts of B cells in CSF correlate with the appearance of new MRI lesions and quicker disease progression (Cepok *et al.*, 2001; Kuenz *et al.*, 2008), and high levels of CXCL13, a B cell chemoattractant, are associated with worse disease progression (Khademi *et al.*, 2011). Furthermore, evidence of the pathogenic role of B cells has also been found in peripheral blood. Patients with MS have myelin-reactive memory B cells circulating (Harp *et al.*, 2010), and they express high levels of CD80 and CD86 (Bar-Or *et al.*, 2001). As for their cytokine production, B cells from patients with MS secrete less IL-10 and more GM-CSF than B cells from healthy subjects do, and this GM-CSF is able to activate the secretion of IL-12 and IL-6 in T cells, two cytokines that are directly

involved in Th1 and Th17 responses (Li *et al.*, 2015). Interestingly, in patients with MS treated with anti-CD20 therapy, newly-originated B cells were found to secrete reduced amounts of GM-CSF and IL-6 and increased amounts of IL-10 (Barr *et al.*, 2012; Li *et al.*, 2015).

The dominant autoantigen or **epitope** triggering RRMS is unknown (Vaughan *et al.*, 2014), but numerous antigens derived from myelin proteins have been identified as candidate autoantigens for the disease. These include the myelin basic protein (MBP), the myelin oligodendrocyte glycoprotein (MOG), the proteolipid protein (PLP), the myelin-associated glycoprotein, the myelin-associated oligodendrocyte basic protein, the peripheral myelin protein, the myelin protein P0 and the oligodendrocyte-myelin glycoprotein. Interestingly, MOG autoantibodies have been identified in patients with CIS and MS and correlate with the disease course (Di Pauli *et al.*, 2011), although the exact role of autoantibodies in disease pathogenesis remains to be determined. Other structures of the myelin, such as gangliosides, the KIR4.1 subunit of potassium channels and heat shock proteins (HSP) —specifically αB-crystallin, Hsp60 and Hsp70— have also been proposed as candidate autoantigens (Quintana *et al.*, 2008; Sadatipour *et al.*, 1998; Srivastava *et al.*, 2012; van Noort *et al.*, 1995). Specific neuron components could also function as potential autoantigens, with neurofilaments, neurofascin and contactin-2 providing possible mechanisms for axonal and grey matter damage (Huizinga *et al.*, 2008; Mathey *et al.*, 2007; Pröbstel *et al.*, 2015). Furthermore, both intramolecular and intermolecular epitope spreading has been described in patients with MS (Muraro *et al.*, 2003; Tuohy *et al.*, 1997), contributing to the perpetuation and diversification of the autoimmune response. Experts argue that epitope spreading could drive the occurrence of flares (Tuohy and Kinkel, 2000), but this theory cannot be upheld by current evidence on disease progression (Riedhammer and Weissert, 2015).

DCs and macrophages, as professional APCs, are also involved in the pathogenesis of the disease. For instance, **DCs** accumulate in white matter lesions of patients with MS, their frequency in CSF correlating negatively with disease duration (Lande *et al.*, 2008; Pashenkov *et al.*, 2001; Serafini *et al.*, 2006). Also, peripheral blood myeloid DCs (myDCs) from patients with MS have elevated expression of CCR5 (Pashenkov *et al.*, 2002), increased expression of CD80 and CD40 and a polarised Th1 response (Karni *et al.*, 2006). As for plasmacytoid DCs (pDCs), their numbers are increased during exacerbations as compared to patients in remission (Longhini *et al.*, 2011). Following the same pattern, monocyte-derived DCs express higher levels of IFN-γ, TNF-α, and IL-6 than their counterparts from healthy subjects (Huang *et al.*, 1999). Concerning **macrophages**, and especially **microglia**, which are the bone marrow-derived resident macrophages of the CNS, they can present

antigens in an immunogenic manner in the context of inflammation (Fabriek *et al.*, 2005; Jack *et al.*, 2005). Upon immune activation, microglia upregulate the expression of CD45, MHC class I and MHC class II and costimulatory molecules to activate $CD4^+$ and $CD8^+$ T cells (Chastain *et al.*, 2011). Surprisingly, **astrocytes** can also present antigens, aside from their responsibilities in the absorption of neurotransmitters and the maintenance of the BBB. In MS lesions, they have the potential to upregulate class II HLA molecules and costimulatory molecules (Zeinstra *et al.*, 2000, 2003), and they secrete chemokines to attract other components of the immune system and activate microglial antigen presentation (Chastain *et al.*, 2011). By contrast, astrocytes can also produce TGF-β and IL-10 to downregulate microglial activation and MHC class II expression (Aloisi *et al.*, 2000). Hence, their final role in MS disease pathogenesis or protection remains to be wholly established.

As for the role of Treg lymphocytes, both classical $FoxP3^+$ Treg cells and TR1 are believed to be important in MS. As it was the case in T1D, studies in patients with MS have been unable to report differences in the frequency of **$FoxP3^+$ Treg cells** in blood, but authors have described that Treg cells from patients with MS have reduced suppressive capacity and that effector T cells are resistant to Treg cell-mediated suppression (Bhela *et al.*, 2015; Costantino *et al.*, 2008). Supporting both findings, genes coding for molecules related to Treg cell function, such as CD25, CD127 and CD58, have been identified as risk alleles for MS predisposition (Procaccini *et al.*, 2015), and an elevated *IL6RA* gene expression and IL-6 signalling has been found in $CD4^+$ T cells of patients with RRMS (Schneider *et al.*, 2013). Moreover, during the remission phase of MS, circulating $CD4^+CD25^+$ Treg cells display higher FoxP3 expression (Frisullo *et al.*, 2009), and treatment with disease-modifying IFN-β further restores the suppressive function of Treg cells (Korporal *et al.*, 2008). Additionally, in patients with MS, **TR1 cells** express lower levels of IL-10 upon stimulation with CD46, which is a known inductor of IL-10, than their counterparts from healthy individuals (Astier *et al.*, 2006). In fact, IL-10 secretion by TR1 cells is associated with decreased disease activity, and those patients who respond to IFN-β therapy possess TR1 cells with enhanced expression of IL-10 and CD46 (Chiarini *et al.*, 2012). Regarding **$CD52^+$ Treg cells**, they could have a dual role in the disease pathogenesis. On the one side, depletion of these cells by alemtuzumab treatment decreases the frequency of relapses in patients with MS (Coles *et al.*, 2012), but on the other side, the emergence of new autoimmune diseases stands as a remarkable side effect of this treatment and points to the significant autoimmunity-blocking capacity of these cells (Bandala-Sanchez *et al.*, 2013). Antigen-specific **$CD8^+$ Treg lymphocytes** are also known to participate in MS. This subset has the capacity to kill pathogenic $CD4^+$ T cells and interact with DCs to suppress $CD4^+$ T cell proliferation. It is interesting that, during relapses, these suppressive abilities are reduced

(Baughman *et al.*, 2011). Finally, **NK cells** are believed to play an immunoregulatory role in the disease, although they have been found in demyelinating lesions (Traugott, 1985). For instance, $CD56^{bright}$ cells from untreated patients display a reduced ability to inhibit the proliferation of activated T cells (Laroni *et al.*, 2016), perhaps because T cells from patients express high levels of the NK-inhibitory ligand HLA-E or low levels of NK-activating ligand CD155 (Gross *et al.*, 2016). Supporting this, daclizumab treatment increases the expression of CD155 on $CD4^+$ T cells from patients and improves NK suppression capacity. Furthermore, a reduced NK frequency has been associated with relapses in MS (Morandi *et al.*, 2008), and patients treated with daclizumab or IFN-β display an expansion in the $CD56^{bright}$ NK cell compartment (Bielekova *et al.*, 2006; Saraste *et al.*, 2007).

3.4.2. Rodent experimental models in MS

In the study of MS, animal models have been employed to study the pathogenic mechanisms of the disease and to test novel therapeutic approaches. The most frequently used animal models are described in this section.

The most commonly studied animal model of MS is the **experimental autoimmune/allergic encephalomyelitis (EAE)** since it mimics critical features of human disease, such as inflammation, demyelination and gliosis (Vaughan *et al.*, 2014). These models include mice and rats primarily, but rabbits and non-human primates could also be employed (Constantinescu *et al.*, 2011). In mice, the autoimmunity to CNS autoantigens characteristic of EAE is induced through immunisation, whereby the myelin-related antigenic peptide is emulsified in complete Freund adjuvant to enhance the peripheral immune response and the breach of immunological tolerance (Bjelobaba *et al.*, 2018). Moreover, the immunisation requires the additional injection of pertussis toxin on the day of immunisation and 48 hours later (Stromnes and Goverman, 2006), which is thought to facilitate immune cell entry into the CNS and promote proliferation and cytokine production by T cells (Bjelobaba *et al.*, 2018). EAE is characterised by an ascending paralysis beginning at the tail that progresses to the limbs, and can be induced in mice with different genetic background with differing resulting courses (Procaccini *et al.*, 2015). For instance, the immunisation with the immunodominant epitope of PLP ($PLP_{139-151}$) to SJL/J mice induces a relapsing-remitting (RR) disease course (Tuohy *et al.*, 1989), whereas the immunisation with the immunodominant epitope of MOG protein (MOG_{35-55}) induces an EAE disease that is chronic in nature (Tompkins *et al.*, 2002). Of note, the latter has aided in the elucidation of the role of several immunologic components in the experimental and human diseases, such as $CD8^+$ T cells (Koh *et al.*, 1992), $CD4^+$ T cells (Baron *et al.*, 1993), B cells (Hjelmström *et al.*, 1998) and monocytes (Bullard *et al.*, 2007).

In EAE, experts considered that Th1 cells were the effector $CD4^+$ T cells driving the autoimmune response (Sospedra and Martin, 2005), but recent evidence indicated that IFN-γ is not required for EAE induction and that it may even have a suppressive activity when administered to these mice (Berghmans *et al.*, 2006; Wensky *et al.*, 2005). Supporting this hypothesis, the course of EAE in IFN-γ knockout mice and in mice administered with anti-IFN-γ antibodies is worse (Billiau *et al.*, 1988; Ferber *et al.*, 1996). As for IL-17, knockout mouse models for the *Il17* or *Il17r* genes show a reduced incidence, severity and delayed onset of EAE (Hu *et al.*, 2010); therefore, IL-17 is believed to be a central mediator of autoimmunity in this model. It is worth mentioning that, in humans, the production of either cytokine is associated with MS (Lock *et al.*, 2002), as described in section 3.4.1. Regarding autoantigens, MS and EAE share several targets, such as the MBP, MOG and PLP (Sospedra and Martin, 2005). Interestingly, in the context of HLA class II alleles that confer risk of MS, immunodominant regions of MBP show overlap with those that can induce the disease in susceptible rodent and non-human primate models (Rao and Segal, 2012; Sospedra and Martin, 2005). $CD4^+$ T cells are known to be the predominant effector cells in EAE (Kroenke *et al.*, 2008) but, to date, very few studies have addressed the role of $CD8^+$ T cells and B cells, proven as crucial players in MS pathogenesis (Procaccini *et al.*, 2015).

Other experimental models are **virally-induced**, exemplified by the Theiler's murine encephalomyelitis virus (TMEV) infection. TMEV is a non-enveloped single-stranded RNA virus of the picornavirus family with neurotropism (Tsunoda and Fujinami, 2010), which induces an inflammatory demyelinating disease in mice that is chronic-progressive in nature (Owens, 2006). It has allowed for the study of axonal damage and inflammatory-induced demyelination. **Toxin-induced models**, such as the cuprizone-supplemented diet fed to C57BL/6 mice or the injection of lysolecithin to SJL/J mice, have permitted for the study of demyelination and remyelination processes (Procaccini *et al.*, 2015).

Therefore, since these models do not perfectly reproduce all the aspects of the human disease, experts are debating their usefulness. In particular, the main differences between experimental models and human disease are that the former are genetically identical and develop the disease through active immunisation. Nevertheless, experimental models currently stand as a significant tool to study all aspects of pathogenesis and treatments that cannot be studied in humans.

4. Immunotherapies to tackle autoimmune diseases

Today, the incidence of autoimmune diseases is increasing at a dramatic pace. Throughout all the vast spectrum of autoimmune diseases, afflicted patients still require the administration of immunomodulatory drugs or hormonal replacement thera-

py for the rest of their lives, converting these diseases into chronic and manageable at best. In many patients, however, they are debilitating, eventually fatal, and available treatments are not without severe adverse effects. Currently, these therapies are not targeting the fundamental cause of the disease, which is a breach of tolerance to self-antigens. These last years, however, several approaches tackling the root of the problem have emerged, and clinical trials exploring their safety, feasibility and efficacy are now a reality. In this section, they are divided into systemic therapies, cell-based therapies and antigen-specific therapies, which include DNA-based, protein-based and peptide-based, nanoparticle-based and lipid nanovesicle-based strategies (**Figure 6**).

4.1. Systemic therapies

Systemic therapies are used with the objective to suppress general pathways of the immune response. In MS, for instance, the available armamentarium of therapeutic drugs stems from the clinical benefit they have demonstrated in clinical trials (Zhang *et al.*, 2018). The first trial assessed the effects of using adrenocorticotropic hormone (ACHT) during MS flares to improve neurological recovery, suggesting only modest beneficial effects but emerging as the gold-standard treatment of MS relapses (Rose *et al.*, 1970). In the 1990s, a 5-year placebo-controlled trial testing IFN-β1b treatment showed a significant reduction in MS relapse rate and new lesion development (Paty and Li, 1993), which led to its approval in 1993 (Zhang *et al.*, 2018). Subsequently, other disease-modifying drugs such as glatiramer acetate (Comi *et al.*, 2001; Johnson *et al.*, 1995, approved in 1997), mitoxantrone (Hartung *et al.*, 2002; Millefiorini *et al.*, 1997, approved in 2000), natalizumab (Miller *et al.*, 2003, approved in 2004), fingolimod (Kappos *et al.*, 2010, approved in 2010), teriflunomide (O'Connor *et al.*, 2011, approved in 2012), dimethyl fumarate (Kappos *et al.*, 2008, approved in 2013), alemtuzumab (Coles *et al.*, 2012, approved in 2014), daclizumab (Bielekova *et al.*, 2009, approved in 2016), ocrelizumab (Kappos *et al.*, 2011; Montalban *et al.*, 2017, approved in 2017) and cladribine (Giovannoni *et al.*, 2010, approved in 2017) solidly demonstrated a therapeutic benefit, albeit mild and severe adverse effects also manifested. Moreover, modified formulations of said treatments have made their way into the clinics, such as pegylated interferon, which requires less frequent dosing than available IFN-β treatment (Pearson *et al.*, 2016, approved in 2014). Unquestionably, the last two decades have been pivotal in changing the therapeutic landscape of MS and have provided a broad range of treatments that have converted MS into a chronic and, under the most favourable circumstances, even manageable disease.

Other general immunomodulatory procedures have been tried in MS. Bone marrow transplantation and immune reconstitution have proved to suppress relapses and slow disease progression, but the drawbacks of the global immune suppression

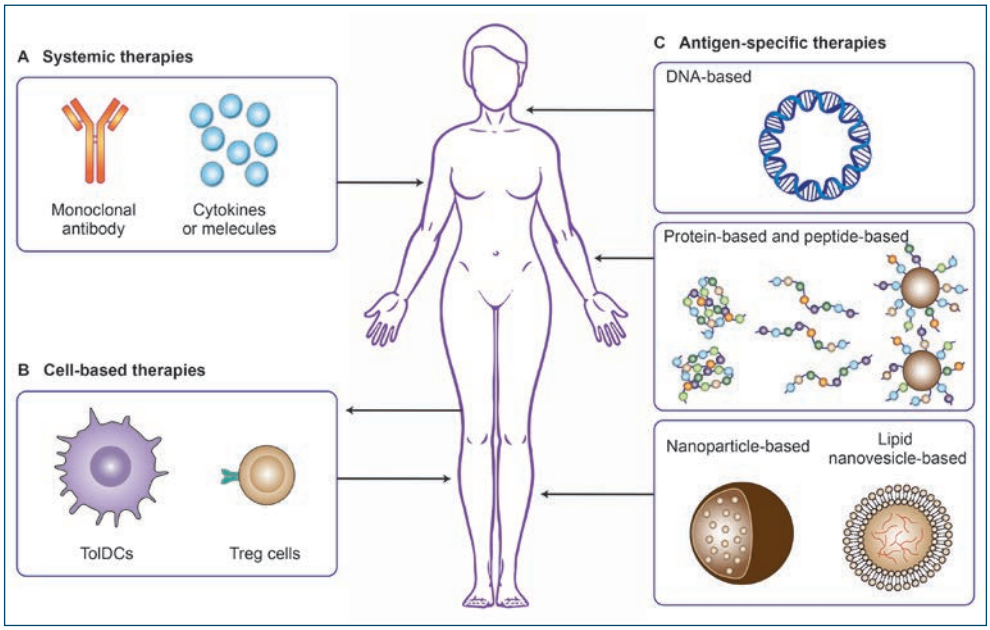


Figure 6. ***Current approaches in the field of tolerance-restoring immunotherapies for autoimmune diseases.*** **(A)** *Systemic therapies, which are mainly based on the use of monoclonal antibodies or molecules targeting general mechanisms of the immune system.* **(B)** *Cell-based therapies, such as tolDCs and Treg cells.* **(C)** *Antigen-specific therapies, represented by DNA-based, protein-based and peptide-based, nanoparticle-based and lipid nanovesicle-based strategies. Adapted from Wraith, 2018; infographic by VRB and SRF.*

required for transplantation may outweigh the benefits of this therapy (Burt *et al.*, 2015; Daikeler *et al.*, 2012; Fassas *et al.*, 1997). Furthermore, intravenous immunoglobulin infusion and plasmapheresis have exhibited a beneficial effect as well as adverse events, but the mechanisms involved are yet to be fully understood (Sorensen *et al.*, 2002; Weinshenker, 2001).

In T1D, however, clinical trials using systemic therapies have not been as successful or, at least, have not surpassed the clinical benefit that exogenous insulin administration has demonstrated over decades. Back in the early 1980s, cyclosporin A provided the first evidence that the modulation of the immune response had therapeutic value in T1D, and even more so when the treatment achieved insulin independence (Feutren *et al.*, 1986; Stiller *et al.*, 1984). However, continuous treatment with cyclosporin had severe toxic effects associated, and when discontinued, endogenous insulin production returned to baseline levels, two facts that determined its discard (Parving *et al.*, 1999). In the 1990s, therapeutics targeting the CD3 molecule such as teplizumab and otelixizumab significantly improved C-peptide levels and metabolic control (Hagopian *et al.*, 2013; Keymeulen *et al.*, 2010), probably by the transient reduction in circulating T cells they cause, but with detectable adverse

effects (Daifotis *et al.*, 2013). Therapeutics targeting other immune-related molecules have been tested in T1D, such as rituximab (anti-CD20, Pescovitz *et al.*, 2009), abatacept (CTLA-4 agonist, Orban *et al.*, 2011) and alefacept (anti-CD2, Rigby *et al.*, 2016), obtaining similar results. Moreover, cytokines and cytokine-targeting biotherapeutics have been employed in T1D clinical trials. Low-dose IL-2 therapy was well-tolerated and induced an increase in regulatory cytokines levels in plasma, along with augmented frequencies of $CD4^+FoxP3^+$ and $CD8^+FoxP3^+$ Treg cells (Hartemann *et al.*, 2013; Rosenzwajg *et al.*, 2015). Glucose control was not improved in this intervention, but this matter will be addressed in a subsequent clinical trial (NCT02411253). Etanercept, a recombinant TNF-α antagonist, increased C-peptide production and improved metabolic control in a pilot study (Mastrandrea *et al.*, 2009), but larger studies have not been conducted to date. By contrast, anakinra (IL-1 receptor antagonist) and canakinumab (anti-IL-1β) monoclonal antibodies failed to preserve β-cell function (Moran *et al.*, 2013). Of note, efforts made by the Type 1 Diabetes TrialNET (Skyler *et al.*, 2008, www.trialnet.org) and the Immune Tolerance Network (Bluestone and Matthe, 2001, www.immunetolerance.org) in the last years have significantly contributed to trial development in the field of T1D.

Other miscellaneous immunotherapies have also been tested in patients with T1D. The anti-lymphocyte serum or anti-thymocyte globulin, which is a concentrate of immunoglobulins directed against several T and B cell receptors, was used on newly-diagnosed patients (Saudek *et al.*, 2004). Even though the authors reported complete diabetes remission in a few treated patients, the results of subsequent trials have not been concordant (Gitelman *et al.*, 2013, 2016). Recently, low-dose anti-thymocyte globulin has been combined with GM-CSF without any additional beneficial effect (Haller *et al.*, 2015, 2018). Furthermore, based on the immunomodulatory effects of vitamin D, patients with T1D at recent onset have been treated with cholecalciferol or calcitriol, but the resulting improvements in HbA1c, C-peptide levels and insulin requirement differed amongst them (Bizzarri *et al.*, 2010; Gabbay *et al.*, 2012; Pitocco *et al.*, 2006; Walter *et al.*, 2010).

4.2. Cell-based therapies

Accumulating knowledge in the field has paved the way for the emergence of tolerance-inducing cell-therapies, in which experimental models have played a crucial role by bringing researchers closer to the fundamental problem underpinning autoimmunity (Stojanovic *et al.*, 2017; ten Brinke *et al.*, 2019). Both APCs and T cells with regulatory capacity can be obtained from patients, modulated and expanded *ex vivo* to attain sufficient numbers prior to reinfusion and, importantly, in an autologous manner. However, this kind of cell-therapy involves expensive production under

good manufacturing practices. Furthermore, numerous procedures and protocols have been implemented to generate tolDCs and Treg cells, countless patterns of administration —route, number of cells, frequency of administration— have been used by researches in the field, and mechanisms of actions are not always described thoroughly. Therefore, the standardisation of tolerogenic cell therapy has become imperative in the last years (Fuchs *et al.*, 2017; ten Brinke *et al.*, 2015).

APCs such as DCs, which are capable of orchestrating the immune response, have been rendered tolerogenic by various mechanisms with the aim to re-educate the mistaken autoimmune recognition. In human, tolDCs are usually differentiated from monocytes, which are readily and largely available in peripheral blood, in a relatively short period of time (Chapuis *et al.*, 1997). Broadly speaking, clinical trials using this approach have primarily focused on demonstrating the safety of this procedure, and efficacy will be thoroughly measured in subsequent trials. In T1D, in a proof-of-concept phase 1 trial, tolDCs were generated with anti-sense oligonucleotides targeting CD40, CD80 and CD86 transcripts and injected intradermally (i.d.) in the upper abdominal area that overlays the location of the pancreas (NCT00445913, Giannoukakis *et al.*, 2011). Researchers argued that prior DC pulsing with self-peptides to direct the tolerance re-establishment could hinder the effectiveness of tolDCs treatment, since patients with T1D do not display uniform self-autoreactivity and antigen spreading is an inevitable occurrence in the disease (Phillips *et al.*, 2019). Thus, the tolDCs would travel to the pancreas and associated PLNs to take up relevant β-cell autoantigens, which they would present in a tolerogenic manner since the expression of costimulatory molecules had been repressed. In this study, patients with established disease for longer than five years were the recipients of this therapy, which was safe and well-tolerated, but a phase 2 of this trial in newly-diagnosed patients is now being conducted (NCT02354911). Another phase 1 trial in T1D has tested the safety of injecting i.d. vitamin D3 and dexamethasone-induced tolDCs pulsed with a C19-A3 proinsulin peptide (NTR5542), although results are yet to be reported. In MS, tolDCs induced by dexamethasone and pulsed with seven relevant peptides from the proteins MBP, MOG and PLP were injected intravenously (i.v.) to 8 patients in a phase 1b study (NCT02283671, Zubizarreta *et al.*, 2019). The authors reported that the therapy was well-tolerated and preserved clinical stability in the patients, and highlighted an increase in the frequency of TR1 cells in their circulation and higher IL-10 production by their PBMCs after peptide stimulation. On the same note, vitamin D3-generated tolDCs pulsed with myelin-derived peptides will be administered by two different routes —i.d. (NCT02618902) and intranodally (NCT02903537)— in a multi-centre clinical trial.

Autoantigen-pulsed tolDCs therapy has also been tried in other autoimmune diseases. For instance, tolDCs were generated with NF-κB inhibitor Bay11-7082, pulsed with four citrullinated peptides and injected i.d. to HLA-risk positive patients with RA

(ACTRN12610000373077, Benham *et al.*, 2015). This strategy achieved a decrease in effector T cells and pro-inflammatory cytokines and chemokines, although mild and transient adverse events derived from the administration were also present. Other clinical trials have been conducted, with safety assessment as the primary outcome. For instance, tolDCs exposed to dexamethasone and vitamin D3 and pulsed with autologous synovial fluid were transfused intraarticularly without any severe adverse effects (NCT01352858, Bell *et al.*, 2017). Similarly, a still-recruiting trial will administer tolDCs generated in the presence of IFN-α and GM-CSF and tolerised with dexamethasone by intra-articular injection (NCT03337165). Finally, in refractory Crohn's disease, vitamin A and dexamethasone-induced tolDCs were transfused intraperitoneally (i.p.), showing clinical improvement in a few patients and without relevant adverse effects (EudraCT number 2007-003469-42, Jauregui-Amezaga *et al.*, 2015). Subsequently, a new phase 1 trial will evaluate the safety and clinical response of intralesional tolDCs administration (NCT02622763).

Given that $CD4^+$ Treg cells constitute a capital cell subset in the prompting of immunological tolerance, the feasibility of culturing and expanding them *in vitro* along with the safety of their adoptive transfer has been largely studied in the context of autoimmune diseases (ten Brinke *et al.*, 2019). In T1D, clinical trials employing Treg cell therapy have confirmed that it is indeed safe and well tolerated. In Poland, newly-diagnosed paediatric patients received *ex vivo*-expanded autologous polyclonal $CD4^+CD25^{high}CD127^-$ Treg cells, which increased C-peptide levels and lowered insulin requirements to the extent that two children were entirely insulin-independent one year after the infusion (ISRCTN06128462, Marek-Trzonkowska *et al.*, 2012, 2014, 2016). In the United States, the same approach will be tested in adolescents with newly-diagnosed T1D in low-dose and high-dose conditions (NCT02691247). As for adults with T1D, Bluestone and colleagues reported that polyclonal Treg cell therapy achieved the persistence of C-peptide levels for more than two years after the cell transfer (NCT01210664, Bluestone *et al.*, 2015). In a subsequent clinical trial, this group has superimposed two IL-2 courses to polyclonal Treg cell therapy (NCT02772679), but the trial is currently suspended to allow time for additional analysis. Also in T1D, polyclonal Treg cell therapy has been combined with other therapeutic agents. For instance, *ex vivo*-expanded polyclonal Treg cells were combined with anti-CD20 antibody and infused into recent-onset patients with T1D, but results have yet to be reported (EudraCT number 2014-0044319-35). Finally, two trials currently being conducted have administered third-party cord-blood polyclonal Treg cells, alone or in combination with liraglutide, to patients with T1D at recent onset (NCT02932826 and NCT03011021). In MS, only one clinical trial, albeit still in the recruiting phase, will administer *ex vivo*-expanded polyclonal Treg cells (EudraCT number 2014-004320-22) i.v. and intrathecally.

Other autoimmune diseases have also been approached with Treg cell transfer. For instance, i.v. infusion of *in vitro*-expanded autologous ovalbumin-specific TR1 cells ameliorated Crohn's disease (EudraCT number 2006-004712-44, Desreumaux *et al.*, 2012).

4.3. Antigen-specific therapies

Even though systemic therapies and cellular strategies are very well tolerated and show therapeutic value, they are not exempt from the risk of inducing general immunosuppression or jeopardising immunosurveillance mechanisms, two outcomes that could be potentially harmful if not carefully considered. Perhaps, for autoimmune diseases, given that the erroneous recognition is directed against a small number of autoantigens, antigen-specific immunotherapies represent a more suitable approach, since the rest of the immune response would remain unaltered. They are divided into DNA-based strategies, protein-based and peptide-based therapies and nanotechnological strategies.

4.3.1. DNA-based therapies

DNA "vaccination" in autoimmune diseases involves the transient expression of relevant autoantigens using encoded DNA vectors. They are preferably administered intramuscularly (i.m.), as this route allows for more sustained expression of the encoded autoantigen (Serra and Santamaria, 2019). Although further work is required to assess the efficacy and safety of this approach in humans, researches have already tested DNA "vaccination" in MS and T1D. For instance, patients with RRMS were administered i.m. with a DNA vaccine encoding the MBP (BHT-3009, NCT00103974 and NCT00382629), which was well tolerated and reduced the appearance of gadolinium-enhanced lesions, the activation of antigen-specific $CD4^+$ T cells and the levels of myelin-reactive antibodies in the CSF in the following weeks after treatment (Bar-Or *et al.*, 2007; Garren *et al.*, 2008). However, no reductions in the annualised relapse rate at one year were observed, although it was substantially lower than in other trials. As for patients with T1D, the same company administered i.m. plasmid DNA encoding the proinsulin molecule (BHT-3021) to recent-onset patients in a phase 1 trial (NCT00453375), without any detectable adverse events (Roep *et al.*, 2013). This treatment decreased the frequency of peripheral proinsulin-reactive $CD8^+$ T cells and improved C-peptide levels in patients.

In the laboratory, researchers are exploring the tolerogenic potential of the liver. Expression of MBP in this organ generated MBP-specific $FoxP3^+$ T cells in mice and prevented CNS inflammation (Lüth *et al.*, 2008) and, similarly, expression of insulin

B chain 9-23 epitope in NOD mice induced an increase in antigen-specific FoxP3$^+$ T cells, prevented disease development and contributed to its reversal in combination with an anti-CD3 monoclonal antibody (Akbarpour *et al.*, 2015).

4.3.2. Protein-based and peptide-based therapies

A significant number of clinical trials have wagered for administering the whole antigen to induce tolerance. As a case in point, insulin has been given orally (Bonifacio *et al.*, 2015; Chaillous *et al.*, 2000; Krischer *et al.*, 2017; Pozzilli *et al.*, 2000; Skyler *et al.*, 2005; Vehik *et al.*, 2011), intranasally (Fourlanos *et al.*, 2011; Harrison *et al.*, 2004; Näntö-Salonen *et al.*, 2008), s.c. (Diabetes Prevention Trial-Type 1 Diabetes Study Group, 2002) and i.v. (Vandemeulebroucke *et al.*, 2009) but without any beneficial effect in T1D prevention in patients at prediabetes or disease management in patients at recent-onset. However, the Primary Oral Insulin Trial (pre-POINT) observed that insulin-responsive CD4$^+$ Treg cells were expanded after the administration of high doses of oral insulin in children at high risk of T1D (Bonifacio *et al.*, 2015). Since GAD protein is also one of the main autoantigens in T1D, paediatric patients with newly-diagnosed T1D were treated s.c. with GAD combined with aluminium hydroxide (alum) in a phase 2 trial (NCT00435981) and phase 3 trial (NCT0072341), preserving residual insulin secretion (Ludvigsson *et al.*, 2008, 2014), although these results were not replicated in a similar clinical trial (Wherrett *et al.*, 2011). Also, patients with T1D at recent-onset received an i.m. injection of human insulin B-chain in incomplete Freund's adjuvant, eliciting phenotypic and functional Treg cell features in insulin B-chain specific CD4$^+$ T cells without an apparent improvement in β-cell function (NCT00057499, Orban *et al.*, 2010). In MS, a phase 1 trial administering orally bovine MBP yielded no therapeutic benefit, but also no side effects (Weiner *et al.*, 1993).

Naturally-occurring peptides and altered peptides have also been given with the same purpose (Roep *et al.*, 2019). I.d. administration of a HLA-DRB1*0401-restricted proinsulin peptide (C19-A3) to patients with T1D at recent onset proved safe and increased the frequencies of FoxP3$^+$ T cells, decreased the levels of β-cell-specific CD8$^+$ T cells, augmented IL-10 secretion and preserved C-peptide release (Thrower *et al.*, 2009; NCT01536431, Alhadj Ali *et al.*, 2017). When administering s.c. a modified Insulin B 9-23 peptide (NBI-6024) to newly-diagnosed patients with T1D, however, no changes in β-cell function were observed (NCT00873561, Walter *et al.*, 2009). The clinical trials using DiaPep277, an immunodominant epitope of Hsp60, did not report any conclusive effects in terms of efficacy (Huurman *et al.*, 2007; Lazar *et al.*, 2007; Raz *et al.*, 2007; Schloot *et al.*, 2007). In MS, the two first clinical trials administering s.c. altered peptides of MBP_{83-99} resulted in disease worsening as evidenced by MRI scanning and increased MBP reactive T cells in circulation (Bielekova *et al.*, 2000),

and hypersensitivity reactions (Kappos *et al.*, 2000). Afterwards, $DR2^+$ and $DR4^+$ patients with SPMS were treated with an unaltered MBP_{82-98} peptide i.v. in a phase 2 clinical trial, achieving a delay in disease progression (NCT00869726, Warren *et al.*, 2006), but failing to attain the same result in a phase 3 (NCT00468611, Freedman *et al.*, 2011). With the objective to target the epitope spreading process that is thought to drive MS, 20 patients with MS had transdermal application of three myelin peptides (MBP_{85-99}, MOG_{35-55}, and $PLP_{139-155}$), which was well tolerated and induced a significant reduction in the emergence of new gadolinium-enhanced lesions, in relapse rates and in antigen-specific T cell proliferative responses, along with an increase in TR1 cells (Juryńczyk *et al.*, 2010; Walczak *et al.*, 2013). Similarly, a cocktail of four MBP-derived antigenic peptides (ATX-MS-1467) given i.d. to patients with RRMS demonstrated safety (NCT01097668, Streeter *et al.*, 2015) and a decrease in total and new gadolinium-enhanced lesions, as well as a cognitive improvement (NCT01973491, Chataway *et al.*, 2018).

Peptides have also been coupled to other molecules and cells to potentiate their tolerogenic potential. However, no therapeutic effects were described when patients with SPMS received i.v. a peptide derived from MBP (84-102) complexed with MHC class II molecule DR2 (Goodkin *et al.*, 2000), nor when patients with RRMS were administered with MOG_{35-55} complexed with HLA-DR2 (Yadav *et al.*, 2012). Nonetheless, seven myelin peptides (MOG_{1-20}, MOG_{35-55}, MBP_{13-32}, MBP_{83-99}, $MBP_{111-129}$, $MBP_{146-170}$, and $PLP_{139-154}$) were chemically coupled to autologous PBMCs and reinfused to patients with RRMS and SPMS, achieving a decrease in specific T cell responses upon peptide challenge but no detectable clinical benefit (Lutterotti *et al.*, 2013). This antigen-specific tolerance induction might be explained by the release of autoantigen-loaded apoptotic bodies from these PBMCs, which are known to promote self-tolerance. Indeed, the mechanisms involved in this process have been dissected precisely in experimental models of MS (Getts *et al.*, 2011), T1D (Prasad *et al.*, 2012), allergy (Smarr *et al.*, 2011) and transplantation (Luo *et al.*, 2008). In these models, peptide antigens were cross-linked to the surface of splenic leukocytes using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (ECDI), a compound that is known to cause apoptosis, and infused i.v. into the mice. Upon phagocytosis of these cells, APCs promoted antigen-specific immune tolerance by inducing clonal anergy and Treg cells expansion. In this sense, disease-relevant autoantigens have also been coupled to erythrocytes with the objective that they deliver antigen cargo to APCs present in the spleen, which are responsible for the clearance of apoptotic erythrocytes. This approach triggers the deletion of cognate T cells and has demonstrated efficacy in a monoclonal $CD4^+$ TCR transgenic variant of the NOD model (Kontos *et al.*, 2013), the wild-type NOD mice and the EAE-induced experimental model (Pishesha *et al.*, 2017).

4.3.3. Nanoparticle-based strategies

In the last years, pioneering approaches based on nanotechnology have broken through in the field of autoimmunity and tolerance re-establishment (Serra and Santamaria, 2019). The term nanotechnology refers to those agents that, functioning as a unit, display an overall size range of 1-1,000 nm. Albeit still in the experimental or preclinical phase, these nanoparticles have demonstrated potential to act as vehicles for antigen delivery to APCs and as effectors of immunoregulation.

For instance, a refinement of the coupling of antigens to splenic leukocytes or erythrocytes consists of poly(lactic-co-glycolic acid) (PLGA) nanoparticles coupled to disease-relevant antigens. Specifically, myelin antigen-coupled PLGA nanoparticles prevented the onset and alleviated the course of EAE disease when captured by splenic macrophages, which induced clonal T cell anergy and expansion of Treg cells (Getts *et al.*, 2012). Similarly, other authors reported that the same strategy halted the progression of EAE disease by reducing the CNS infiltration of Th1 and Th17 cells (Hunter *et al.*, 2014). Also, PLGA nanoparticles encapsulating rapamycin and autoantigens protected EAE-induced mice from developing the disease through the expansion of antigen-specific Treg cells (LaMothe *et al.*, 2018), and the same approach halted the production of antibodies against coagulation factor VIII in a murine model of haemophilia (Maldonado *et al.*, 2015). In this context, Carambia *et al.*, 2015 described the tolerogenic potential of myelin-loaded polymer nanoparticles selectively delivered to hepatic sinusoidal endothelial cells, which prevented EAE and blunted disease progression in strict dependence on $CD4^+FoxP3^+$ Treg cells.

Nanoparticle-based approaches have also been tested in experimental T1D with successful results. As a case in point, nanoparticles encapsulating proinsulin and the ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) of the aryl hydrocarbon receptor, which has tolerogenic properties, completely suppressed diabetes development in NOD mice (Yeste *et al.*, 2016). This was achieved by the generation of tolDCs —induced by Socs2 activation and subsequent NF-κB inhibition— and expansion of $FoxP3^+$ Treg cells. Moreover, peptide-MHC (pMHC) class I and class II complexes have been coupled to iron-based nanoparticles to tackle both the $CD4^+$ and the $CD8^+$ arms of the autoimmune response (Clemente-Casares *et al.*, 2016; Tsai *et al.*, 2010), with their potential confirmed in experimental models of T1D, MS, arthritis. Authors argue that, albeit similar in nature, pMHC class I and pMHC class II nanoparticles work through different mechanisms: while the first induce the differentiation of naturally-occurring antigen-specific low-avidity $CD8^+$ T cells into memory-like autoregulatory $CD8^+$ T cells, the second trigger the conversion of cognate autoreactive $CD4^+$ T cells into disease-suppressing antigen-specific TR1 cells and recruit regulatory B lymphocytes to the draining lymph nodes (Serra and Santamaria, 2019).

4.3.4. Lipid nanovesicle-based strategies as vehicles

Liposomes, which are spherical vesicles composed of a lipid bilayer with an internal aqueous cavity, have been exploited in autoimmunity as well. In fact, liposomes pose as advantageous over other nanoparticle-based strategies because they are easily customisable and adaptable, inexpensively large-scale produced, standardisable, easy to administer, biocompatible and biodegradable. Contrary to nanoparticles, different liposomal formulations have reached the clinical setting, demonstrating safety and efficacy when acting as drug deliverers (Bulbake *et al.*, 2017). Different formulations are currently approved in cancer therapy, photodynamic therapy, fungal diseases, analgesic therapy and viral vaccination.

In the autoimmunity setting, however, liposomes have only been tested in experimental models yet. For instance, liposomal nanoparticles displaying glycan ligands of the inhibitory coreceptor CD22 and factor VIII caused selective B cell apoptosis and prevented the development of anti-factor VIII antibodies in a murine model of haemophilia (Macauley *et al.*, 2013). Moreover, phosphatidylcholine (PC) liposomes loaded with antigen and the NF-κB inhibitor Bay11-7082 suppressed effector T cells responses and inflammatory arthritis development in a mouse model through the generation of tolDCs and the induction of antigen-specific FoxP3^{+} Treg cells (Capini *et al.*, 2009).

4.3.5. Liposomes exploiting apoptotic mimicry

Given the pivotal importance of apoptotic cell clearing (efferocytosis) in the induction of peripheral tolerance, liposomes have been designed to resemble apoptotic cells with the aim to tackle various tolerance-requiring processes. The fact that DCs acquire tolerogenic features after phagocytosis of apoptotic cells is widely established (Ariel and Ravichandran, 2016; Vives-Pi *et al.*, 2015) and, in the bench, several authors have harnessed the tolerogenic potential of apoptotic cell-induced DCs. Specifically, in T1D, transfusion of apoptotic islet cells arrested the development of diabetes in NOD mice (Xia *et al.*, 2007). Also, in experimental transplantation, the administration of donor apoptotic cells prolonged the survival of cardiac allograft and restrained allorecognition (Wang *et al.*, 2006, 2009; Xu *et al.*, 2004). Hence, with this idea in mind, authors have used PS-rich liposomes. These lipid nanovesicles resemble apoptotic cells in their ability to induce tolerogenic potential in DCs, whereby they halt the *in vitro* maturation of DCs, impair their ability to stimulate T cell proliferation (Chen *et al.*, 2004; Shi *et al.*, 2007) and induce the secretion of anti-inflammatory mediators in phagocytes (Otsuka *et al.*, 2004; Wu and Nakanishi, 2011; Zhang *et al.*, 2006). Importantly, they have demonstrated their potential in the preclinical setting.

As a case in point, PS-liposomes reduced carrageenan-induced paw oedema through a mechanism dependent on peroxisome proliferator-activated receptor (PPAR) pathway activation (Ramos *et al.*, 2007) and improved myocardial infarction repair through the induction of a reparative state in cardiac macrophages (Harel-Adar *et al.*, 2011).

In this context, Marin-Gallen *et al.*, 2010 demonstrated that murine DCs from NOD mice were rendered tolerogenic after *in vitro* engulfment of apoptotic β-cells obtained from NIT-1 cells, an insulinoma line of the same mouse strain. Specifically, these pulsed DCs expressed low levels of costimulatory molecules CD40 and CD86, even after activation with LPS, were impaired to induce autologous T cell proliferation and secreted little amounts of pro-inflammatory cytokines (Marin-Gallen *et al.*, 2010; Pujol-Autonell *et al.*, 2013). One of the hallmarks of efferocytosis by these DCs was the release of PGE_2, which is believed to be partly responsible for the acquisition of immunosuppressive ability (Pujol-Autonell *et al.*, 2013). Furthermore, when these tolDCs were transferred to prediabetic NOD mice, they were capable of arresting autoimmune β-cell destruction, preventing the development of the disease and diminishing the severity of insulitis in pancreatic islets (Marin-Gallen *et al.*, 2010). Importantly, tolDCs loaded with apoptotic cells from an irrelevant cellular line were not able to restore tolerance in NOD mice.

However, when aiming for translationality, it would be impossible to find a substantial source of autologous apoptotic β-cells in humans to implement this therapy. An alternative source of apoptotic β-cells was needed, and that is why authors drew inspiration from nature to devise a synthetic immunotherapy imitating apoptotic β-cells, which consisted of β-cell autoantigen-loaded multivesicular and multilamellar PS-rich liposomes (Pujol-Autonell *et al.*, 2015). In this study, the authors showed that DCs from NOD mice phagocyted PS-liposomes rapidly and efficiently, reaching 75% of capture after only 5 minutes of coculture, and without affecting the viability. Also, the phagocytosis of PS-liposomes loaded with insulin peptides induced a tolerogenic phenotype in DCs, as demonstrated by low expression of MHC class I, MHC class II, CD40 and CD86 molecules, and prompted the secretion of anti-inflammatory mediators like PGE_2. When DCs loaded with these particular liposomes were cocultured with autoreactive T lymphocytes, the results showed that DCs had their ability to induce T cell proliferation and to secrete pro-inflammatory cytokines like IL-17A and IFN-γ impaired in comparison to DCs only exposed to a pro-inflammatory stimulus. Interestingly, when administered to prediabetic NOD mice, these PS-liposomes travelled preferentially to the pancreas and draining lymph nodes. Importantly, these PS-liposomes filled with insulin peptides arrested the autoimmune aggression to β-cells in prediabetic NOD mice, thus preventing the development of overt hyperglycaemia and decreasing the incidence of the disease, and reducing the severity of the insulitis. Empty PS-liposomes, however, did not replicate these re-

sults, hence demonstrating that the tolerance re-establishment prompted by PS-liposomes worked in an antigen-specific manner in this model. Furthermore, the authors demonstrated that antigen-specific $CD4^+$ T cells were expanded in treated NOD mice and that this expansion contained both $FoxP3^+$ and $FoxP3^-$ cells. Therefore, the authors demonstrated that apoptotic mimicry is indeed a powerful tool to restore tolerance to autoantigens, and this is achieved by unsophisticated but clever autoantigen-loaded PS-rich liposomes that co-deliver a binary signal of tolerance and specificity to arrest the undesired autoimmune reaction in a synergistic, effective and safe manner. This technology has been patented (WO2015107140) and licensed to Ahead Therapeutics, SL.

Although further research is required, the immunotherapy consisting of autoantigen-loaded PS-rich liposomes possesses five characteristics that are crucial in its functioning as an antigen-specific tolerogenic agent:

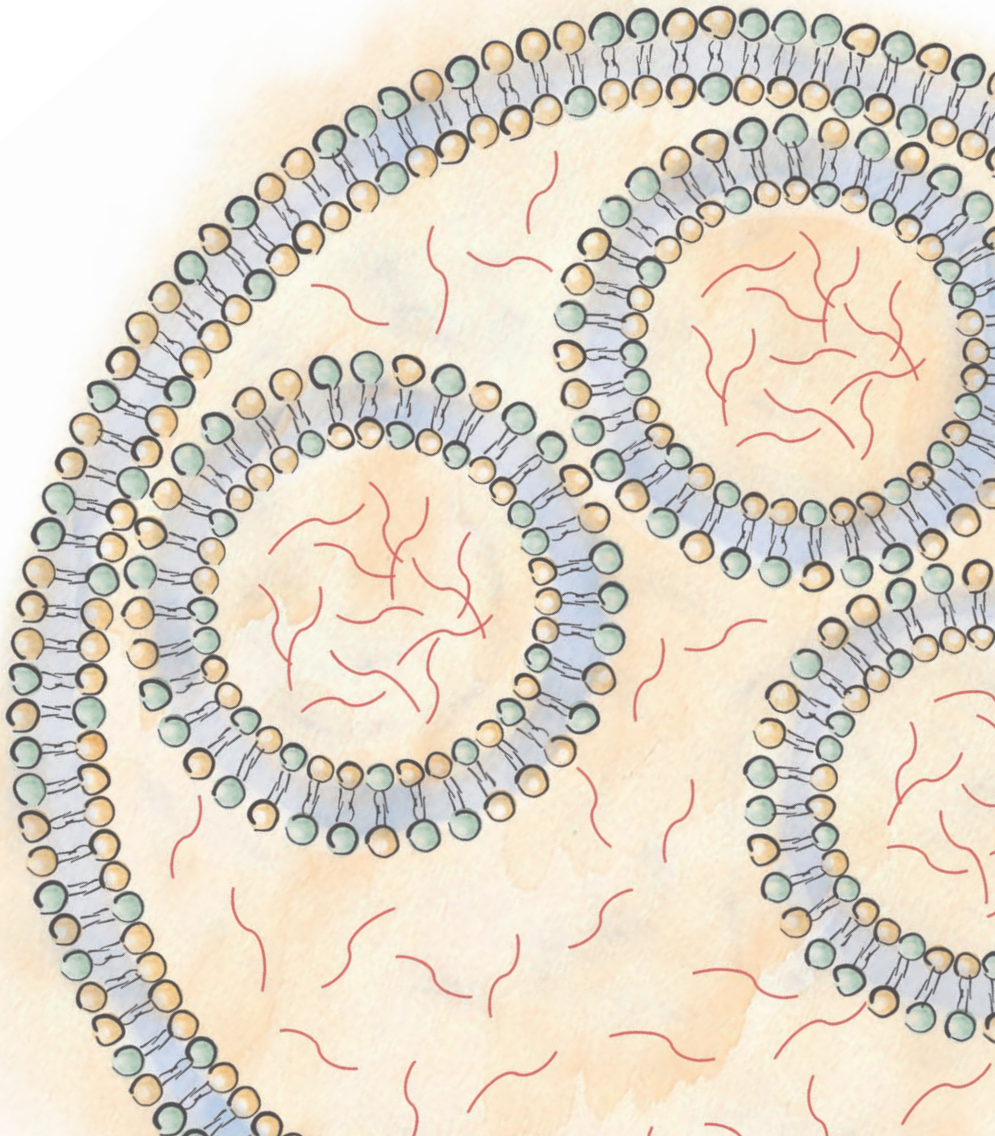
- Firstly, particle size commands whether a particle is internalised by phagocytosis or by cell fusion. Indeed, phagocytosis is the mechanism by which phagocytes engulf particles larger than 500 nm (Oh and Park, 2014), with smaller particles being less efficiently phagocyted (Fang *et al.*, 2006). Also, particle size directly influences the immunogenicity of the particle, as demonstrated by smaller particles (<100 nm) being more inclined to promote $CD4^+$ and $CD8^+$ T cell responses than larger ones (<500 nm) (Fifis *et al.*, 2004).
- Secondly, surface charge (or ζ-potential) can determine whether the particles are phagocyted and the effect they elicit. For example, cationic or anionic particles are more efficiently uptaken than neutral particles (Ahsan *et al.*, 2002; Zahr *et al.*, 2006), and cationic liposomes are more efficient in generating immune responses than anionic or neutral liposomes (Nakanishi *et al.*, 1999).
- Thirdly, the fact that these liposomes are rich in PS targets them to professional phagocytes and cells with functional PS receptors, and provides these liposomes with a unique tolerogenic signal capable of rendering DCs tolerogenic. As aforementioned, PS is one of the main characteristic signals of apoptotic cells, and is sufficient to cause particle engulfment and immunomodulatory responses in phagocytes. As a case in point, while PS-liposomes can resolve inflammation, PS-free liposomes cannot (Ramos *et al.*, 2007). Additionally, PS accompanying antigen administration reportedly induced a tolerogenic immunological memory in experimental models of Pompe disease, haemophilia A and MS in a manner that is strictly dependent on functional PS receptors (Glassman *et al.*, 2018).
- Fourthly, the specific antigen load informs the DCs to promote immune tolerance in an antigen-specific manner without compromising the rest of the immune response. Importantly, this encapsulated antigen load is protected

by the liposome membrane from external degradation until it reaches its site of action.

- Lastly, when compared to naturally-occurring apoptotic cells, PS-rich liposomes have the clear advantage of being inherently unable to degenerate into secondary necrotic cells that could potentially trigger an immunogenic response.

The use of this novel and cost-competitive liposomal immunotherapy in NOD mice has revealed encouraging results, which have pushed the group to delve into its tolerogenic potential and to investigate whether it could act as a platform for restoring tolerance in antigen-specific autoimmune diseases that share the pathological foundation with T1D.

HYPOTHESIS AND OBJECTIVES



1. Hypothesis

To date, virtually no immunotherapies can restore immunological tolerance in a definite manner. In homeostatic conditions, efferocytosis —phagocytosis of apoptotic cells— is one of the mechanisms responsible for maintaining self-tolerance and restraining autoreactivity. By nature's example, we devised a cell-based immunotherapy consisting of dendritic cells rendered tolerogenic after phagocytosis of apoptotic β-cells. This strategy restored antigen-specific tolerance and arrested experimental autoimmune diabetes. In aiming for translationality, however, due to the difficulties in obtaining a substantial source of human autologous apoptotic β-cells, we conceived a synthetic immunotherapy mimicking apoptotic cells, based on phosphatidylserine-rich liposomes encapsulating β-cell autoantigens. Their phagocytosis by dendritic cells also induced the re-establishment of immunological tolerance to β-cells, achieving apoptotic mimicry in a simple, safe and efficient manner. Based on this knowledge, this liposomal immunotherapy constitutes a cutting-edge strategy to recover antigen-specific tolerance in human autoimmune diseases.

The hypothesis of this work is that an antigen-loaded phosphatidylserine-rich liposomal immunotherapy can arrest autoimmunity in several antigen-driven autoimmune diseases through the induction of tolerogenic dendritic cells and the expansion of regulatory T lymphocytes, and that it has the translational potential to tackle human autoimmune diseases.

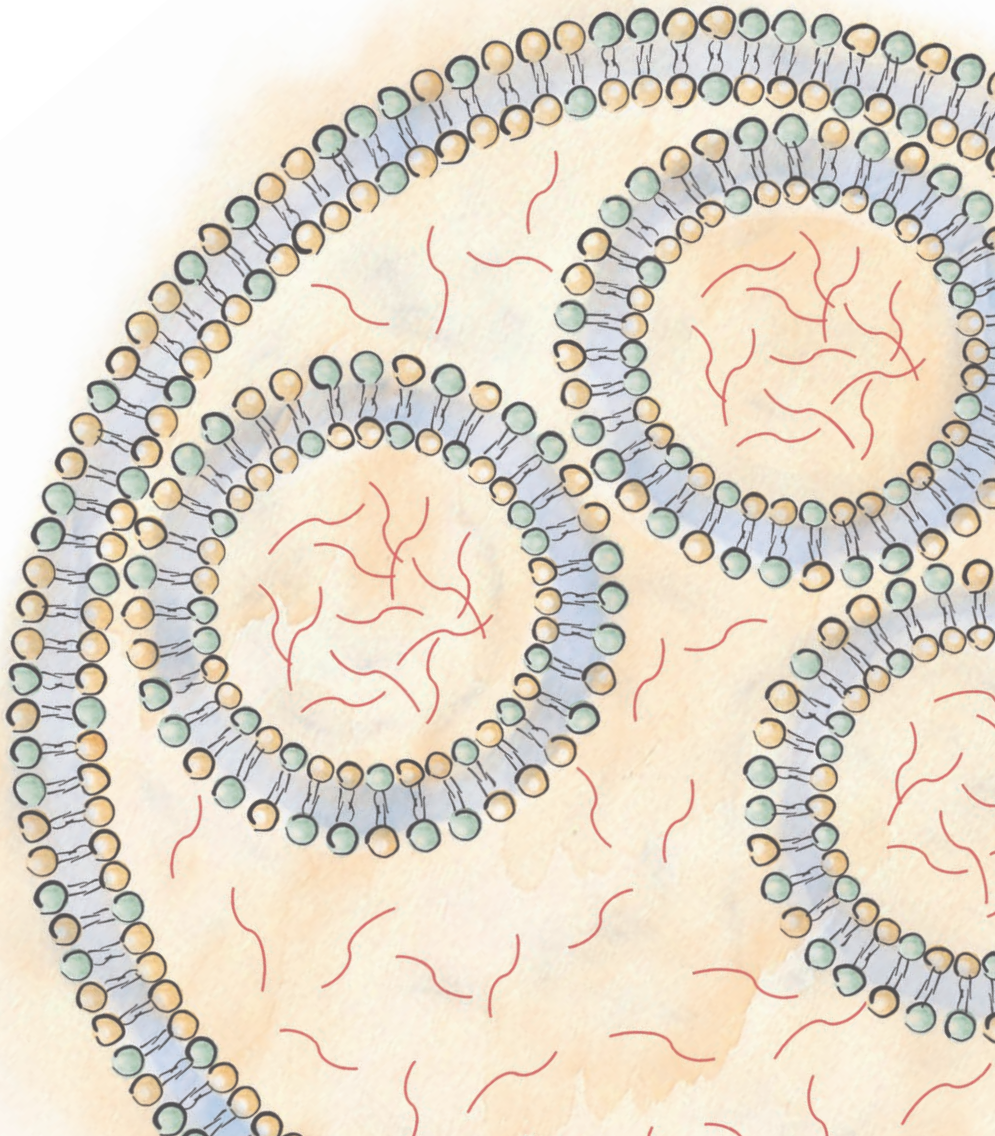
2. Objectives

The main aim of the present work is to globally characterise the tolerogenic potential of a PS-rich liposomal strategy —which is based on the inherent immunomodulating marks of apoptotic cells and thus benefits from the co-delivering of tolerogenic signals and autoantigens— to tackle autoimmunity

To accomplish the main aim, specific objectives were set as follows:

- To assess the nature of the antigen-specific $CD4^+$ T lymphocytes and to identify $CD8^+$ T lymphocytes expanded in NOD mice after PS-liposomes treatment.
- To assess the degree of toxicity and tolerability of the final immunotherapy.
- To determine the immunomodulating and preventive effect of PS-liposomes therapy in experimental MS.
- To define the tolerogenic features acquired by human DCs from adult patients with T1D upon PS-liposomes engulfment, in terms of capture kinetics, phenotype, cytokine secretion, ability to induce autologous proliferation and gene expression.
- To validate the potential of the PS-liposomes strategy to elicit tolerogenic qualities in human DCs from paediatric patients with T1D.

MATERIALS AND METHODS



1. Liposome design

Liposomes were designed and generated to simulate apoptotic cells, as previously described (Pujol-Autonell *et al.*, 2015, 2017; Rodriguez-Fernandez *et al.*, 2018), and protected by intellectual property rights (Patent WO2015107140A1). Thus, they were composed of a lipid membrane and encapsulated autoantigens. In order to adapt the PS-liposomes to the two autoimmune diseases tackled (T1D and MS) and to study the different aspects of the effects they elicit in the immune response, diverse autoantigens were loaded into the liposomes. Finally, liposomes were characterised in terms of diameter, surface charge and peptide encapsulation efficiency.

1.1. Generation of PS-rich liposomes

Liposomes were generated at the Catalan Institute of Nanoscience and Nanotechnology (ICN2, Bellaterra, Spain). Liposomes consisted of 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (PS, Lipoid, Steinhausen, Switzerland), 1,2-didodecanoyl-sn-glycero-3-phosphocholine (PC, Lipoid), and cholesterol (CH, Sigma, Saint Louis, MO, USA). Lipid-conjugated fluorescent dye Oregon Green 488 (OG488) 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE, ThermoFisher Scientific, Waltham, MA, USA) was used to prepare fluorescent liposomes. PS-rich liposomes (PS-liposomes) were prepared using the thin film hydration method from a lipid mixture of PS, PC and CH at 1:1:1.33 molar ratio, respectively, as described (Harel-Adar *et al.*, 2011). Liposomes without PS (PC-liposomes) were generated as controls with PC and CH at 1:1 molar ratio. All liposomes were produced under sterile conditions and at a final lipid concentration of 30 mM, unless otherwise stated. Lipids and fluorescent dyes were dissolved in chloroform and the solvent was removed by evaporation under vacuum and nitrogen. The lipids were hydrated with phosphate buffered saline (PBS, Hyclone, GE Healthcare Life Sciences, Marlborough, MA, USA) in the case of empty liposomes, or with a 0.5 mg/mL solution of the correspondent peptide for loaded liposomes. The liposomes obtained were homogenised to

1 µm by means of an extruder (Lipex Biomembranes, Vancouver, Canada). Information about the liposomes' nomenclature, peptide load and use can be found in **Table 3**.

1.2. Selection of peptides encapsulated within PS-liposomes

Several peptides (**Table 4**) were chosen to fill up liposomes in accordance with the experiments performed (**Table 3**). Each peptide was >95% pure and trifluoroacetic acid was removed. The 2.5mi peptide was dissolved in 0.2% dimethyl sulfoxide (DMSO, Sigma) and further diluted in PBS to reach a concentration of 0.5 mg/mL prior to encapsulation within liposomes. The other peptides were reconstituted with PBS (Hyclone, GE Healthcare Life Sciences) to 0.5 mg/mL.

1.3. Characterisation of PS-liposomes

The particle size of the liposomes, the distribution of the sizes (polydispersity index, PdI) and surface charge —expressed as zeta potential (ζ)— were measured by dy-

Table 3. Liposomes used in the study

Liposome nomenclature	Peptide load (species)	Use
PS-liposomes	None	Control of the effect of antigen load
PS2.5mi-liposomes	2.5mi peptide (mouse)	Detection of 2.5mi^{+}CD4^{+} T cells with A^{g7}/2.5mi peptide MHC class II tetramers
PC2.5mi-liposomes	2.5mi peptide (mouse)	Control of the PS' effect in the detection of 2.5mi^{+}CD4^{+} T cells with A^{g7}/2.5mi peptide MHC class II tetramers
PSNRP-liposomes	NRP-V7 peptide (mouse)	Detection of NRP $^{+}$CD8^{+} T cells with H2K^{d}/NRP-V7 peptide MHC class I tetramers
PCNRP-liposomes	NRP-V7 peptide (mouse)	Control of the PS' effect in the detection of NRP^{+}CD8^{+} T cells with H2K^{d}/NRP-V7 peptide MHC class I tetramers
PS30-liposomes	None	Determination of tolerability (administration pattern assay)
PS90-liposomes	None	Determination of tolerability (posology assay)
PSMOG-liposomes	MOG_{40-55} peptide (mouse)	Preventive effect in MS model
PSA-liposomes	Insulin A chain (human)	Effect in human DCs *in vitro*
PSB-liposomes	Insulin B chain (human)	Effect in human DCs *in vitro*
PSOG488-liposomes	None	Liposome capture kinetics by DCs
PCOG488-liposomes	None	Control of the PS' effect in liposomes capture kinetics by DCs

PS: phosphatidylserine; MHC: major histocompatibility complex; MOG: myelin oligodendrocyte glycoprotein; MS: multiple sclerosis; OG488: oregon green 488; DCs: dendritic cells.

Table 4. Peptides used to load up the liposomes

Name	Species	Residue number	Sequence	Net charge at pH 7	Company
2.5mi peptide	Mouse	11	N-start-AHHPIWARMDA-C-end	0.2	GL Biochem
NRP-V7 peptide	Mouse	9	N-start-KYNKANVFL-C-end	2	GL Biochem
MOG_{40-55} peptide	Mouse	16	N-start-YRSPFSRVVHLYRNGK-C-end	4.1	Immunostep
Insulin A chain	Human	21	N-start-GIVEQCCTSICSLYQLE-NYCN-C-end	–2.3	Genosphere Biotechnologies
Insulin B chain	Human	30	N-start-FVNQHLCGSHLVEA-LYLVCGERGFFYTPKT-C-end	0.1	Genosphere Biotechnologies

MOG: myelin oligodendrocyte glycoprotein.

namic light scattering using Malvern Zetasizer (Malvern Instruments, Malvern, UK) in undiluted samples. Liposome morphology and lamellarity were examined by cryogenic transmission electron microscopy (cryo-TEM) in a JEOL-JEM 1400 microscope (Jeol Ltd., Tokyo, Japan).

Peptide encapsulation efficiencies were calculated according to the equation: encapsulation efficiency (%) = $[(C_{peptide,total} - C_{peptide,out}) / C_{peptide,total}] \times 100$, where $C_{peptide,total}$ is the initial peptide concentration and $C_{peptide,out}$ is the concentration of non-encapsulated peptide. To measure the $C_{peptide,out}$, liposome suspensions were centrifuged at 110,000 × g at 10 °C for 30 minutes. The concentration of non-encapsulated peptide was assessed in supernatants by PIERCE BCA protein assay kit (ThermoFisher Scientific), which profits from the reduction of Cu^{+2} to Cu^{+1} in alkaline medium and the colourimetric reaction of Cu^{+1} when interacting with bicinchoninic acid. This reaction develops linearly with increasing protein concentrations. The assays were carried out in 96-well plates (NUNC Maxisorp 96 well ELISA plate, ThermoFisher Scientific) and standard curves were performed with the same peptide whose encapsulation was being determined. Absorbance was read at 562 nm using a spectrophotometer (Varioskan®, ThermoFisher Scientific) after 1 hour —2 hours in the case of supernatants obtained from liposomes containing 2.5mi peptide— of incubation at 37 °C.

2. Experimental models of autoimmune diseases

Murine models were employed to address the first three objectives of this work. Specifically, to assess the quantity and nature of the antigen-specific T cell response elicited by PS-liposomes treatment and its toxicity *in vivo*, the NOD mouse strain was

used; to validate the preventive effect of the therapy in a second autoimmune disease, the experimental model for MS, the EAE-induced mouse, was utilised.

2.1. Ethics statement

All experiments with animal models were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the *Generalitat de Catalunya*, in the Principles of Laboratory Animal Care by the National Institute of Health, and have followed the principles outlined in the Declaration of Helsinki for animal experimental investigation. Each protocol was approved by the Committee on the Ethics of Animal Experimentation of the Germans Trias i Pujol Research Institute. The permit DAAM for experiments using the animal model of T1D were 8948 and 10030; the one for the experiments using the animal model of MS was 5315.

2.2. NOD mice and monitoring of diabetes onset

Wild-type NOD mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and then kept in the Animal Facility of the Germans Trias i Pujol Institute under SPF conditions. This mouse strain develops autoimmune diabetes spontaneously after 12 weeks of age, and the incidence of diabetes in the colony is 54% in females and 23% in males (Gieras *et al.*, 2017).

The colony was subjected to a 12 hours dark/12 hours light cycle, controlled temperature (20-23 °C) and humidity (34-83%) conditions. Mice were provided with acidic water at pH 5 and irradiated Teklad Global 18% Protein Rodent Diet (Harlan, Indianapolis, IN, USA) *ad libitum*. Glycosuria levels were monitored daily using urine test strips (Menarini, Barcelona, Spain) in order to detect T1D. Diabetes was confirmed when glycaemia raised above 300 mg/dL in one glucotest control (AccuCheck, Roche Diagnostics, Indianapolis, IN, USA) or above 200 mg/dL in two consecutive tests after fasting for 2 hours. Only prediabetic mice were used in the study.

2.3. C57BL/6 mice and induction and monitoring of EAE

Wild-type C57BL/6 inbred mice were obtained from Envigo Rms Spain SL (Sant Feliu de Codines, Barcelona, Spain) and maintained in the Animal Facility of the Germans Trias i Pujol Institute under conventional conditions. Mice were kept in a temperature and humidity-controlled room and subjected to a 12 hours dark/12 hours light cycle. Mice were fed standard Teklad Global 14% Protein Rodent chow diet (Envigo) *ad libitum* alongside autoclaved tap water.

EAE was induced only in 8 week old C57BL/6 female mice. Prior to immunisation procedure, mice were anaesthetised with ketamine/xylazine at 50 and 5 mg/kg body weight, respectively. After confirming that mice had reached an optimal surgical plane of anaesthesia, they received an s.c. injection of 50 µg MOG_{40-55} peptide (Immunostep, Salamanca, Spain) diluted in PBS (Hyclone, GE Healthcare Life Sciences) and emulsified in an equal volume of complete Freund's adjuvant, which contained 4 mg/mL of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). This injection was performed in both flanks of the mice. Next, 250 ng of Pertussis toxin (Sigma) was injected i.v. at days 0 and 2. Each animal was weighed and monitored daily for welfare and severity of clinical signs, which were expressed according to the following criteria: 0, asymptomatic; 0.5, loss of distal half of tail tone; 1, loss of entire tail tone; 1.5, hind limb weakness; 2, hind limb paralysis; 2.5, hind limb paraplegia; 3, forelimb weakness; 4, quadriparesis; 4.5, severe quadriparesis; 5, quadriplegia; and 6, death (Mansilla *et al.*, 2015). Clinical follow-up was performed in a blinded manner by two different observers.

3. Human samples

In order to characterise the tolerogenic features acquired by human immune cells after PS-liposomes treatment, its effects were tested in monocyte-derived DCs obtained from peripheral blood of both adult patients and paediatric patients with T1D and from control subjects.

3.1. Ethics statement

The studies with human cells were approved by the Committee on the Ethics of Research of the Germans Trias i Pujol Research Institute and Hospital. The reference number of the studies approved were PI-14-071 for adult subjects and PI-16-083 for paediatric subjects. All experiments were conducted following the principles outlined in the Declaration of Helsinki for human research and in accordance with the approval of the Committee on the Ethics Research of the institution. All study participants (or their legal guardians) gave informed consent. The blood samples obtained from the donors were used on the same day of collection. The collection and processing of personal and clinical data from subjects included in the study were limited to those data which were necessary to achieve the objectives of the study. These data were collected and processed with adequate precautions to ensure confidentiality and compliance with applicable data privacy protection laws and regulations.

3.2. *Blood samples from adult subjects*

Samples of peripheral blood were collected from adult patients with T1D and control subjects. Inclusion criteria for all subjects were 18-55 years of age, a body mass index (BMI) between 18.5 and 30 kg/m^2 and, for patients with T1D, fulfilment of T1D classification criteria and evolution of disease longer than six months. Exclusion criteria were being under immunosuppressive or anti-inflammatory treatment or undergoing pregnancy or breastfeeding. For the RNA-sequencing (RNA-seq) experiment, inclusion criteria were restricted to a maximum BMI of 24.9 kg/m^2, a maximum disease duration of 5 years (in order to minimise the effect that long-term hyperglycaemia could have on genetic and/or epigenetic profiles), and the presence of other chronic diseases became an exclusion criterion.

3.3. *Blood samples from paediatric subjects*

Peripheral blood samples were obtained from paediatric patients with T1D and control subjects. Inclusion criteria for all subjects were 1-18 years of age and normal BMI according to the World Health Organisation (WHO) BMI paediatric growth chart and, in the case of patients with T1D, fulfilling of the classification criteria for T1D. Exclusion criteria were being under immunosuppressive or anti-inflammatory treatment, the presence of other autoimmune diseases, T2D, pregnancy, compromised kidney function or liver diseases.

4. Assessment of the effect of PS-liposomes *in vivo* in experimental models of autoimmune diseases

In this project, the NOD mouse strain and the EAE-induced mouse model were used to study the *in vivo* effects caused by the administration of PS-liposomes. Specifically, treatment with PS-liposomes to NOD mice allowed for the study of the adaptive immune response elicited by the therapy with the use of MHC tetramer staining experiments. The term *tetramer* refers to the multiple antigen-MHC-biotin complexes bound to a fluorescently-labelled streptavidin molecule, which are capable of binding multiple antigen-specific TCRs on a T lymphocyte, thus enabling the detection and quantification of these antigen-specific T cells by flow cytometry (**Figure 7**). Furthermore, the NOD model was employed to determine the degree of tolerability and toxicity of the final PS-liposomes product through the testing of severe posology and administration patterns. On the other hand, the administration of PS-liposomes to EAE-induced mice served to evaluate their effect in arresting the autoimmune response driving experimental MS.

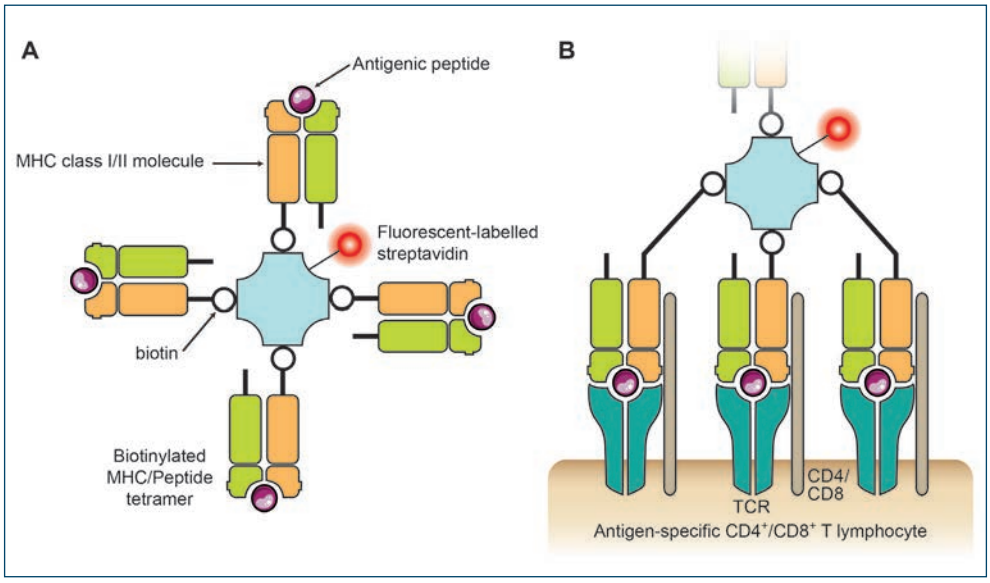


Figure 7. ***Graphical representation of peptide-loaded MHC tetramers.*** **(A)** *A biotinylated MHC class I/II tetramer loaded with an antigenic peptide, connected by fluorescent-labelled streptavidin.* **(B)** *Antigenic peptide/MHC class I/II tetramers bound to the TCRs of antigen-specific CD8⁺/CD4⁺ T lymphocytes, respectively, thus allowing for the tracking of the same cells. Infographic by VRB and SRF.*

4.1. Detection of antigen-specific TR1 CD4⁺ T cells in NOD mice after PS-liposomes treatment

To study changes in CD4⁺ T cell subsets, A^{g7}/2.5mi peptide MHC class II tetramers were used. The 2.5mi peptide (N-start-AHHPIWARMDA-C-end, **Table 4**) is a strong mimotope agonist peptide for the diabetogenic CD4⁺ T lymphocyte clone named BDC-2.5 (Yoshida *et al.*, 2002), which specifically recognises the Chromogranin A molecule (Stadinski *et al.*, 2010). A^{g7}/2.5mi peptide MHC class II tetramers allow for the detection of a naturally-occurring CD4⁺ T cell population, termed 2.5mi⁺ T lymphocytes, which share reactivity with this clone in NOD mice. Thus, to detect 2.5mi⁺CD4⁺ T cells with A^{g7}/2.5mi peptide MHC class II tetramers, female prediabetic 8-14 week old NOD mice were treated i.p. with a single 3.5 mg lipid of PS2.5mi-liposomes dose in 200 µL PBS (Hyclone, GE Healthcare Life Sciences). Control groups were equally administered with either 200 µL PC2.5mi-liposomes or saline solution (PBS, sham). Mice were monitored for glycosuria as abovementioned (see section 2.2) and euthanised by cervical dislocation 4 days after the injection. Spleen, PLN and mediastinal lymph nodes (MDLN) were harvested for further evaluation.

Subsequently, A^{g7}/2.5mi peptide MHC class II tetramers were mounted from MHC-peptide monomers. Both these monomers and control A^{g7}/glucose-6-phos-

phatase isomerase-derived (GPI) peptide monomers were kindly provided by Dr Thomas Stratmann (University of Barcelona). Briefly, soluble A^{g7} molecules connected to the 2.5mi peptide (N-start-AHHPIWARMDA-C-end, GL Biochem, Shanghai, China, **Table 4**) or to the $GPI_{282-292}$ (N-start-LSIALHVGFDH-C-end, GL Biochem) control peptide were expressed in SC2 cells, which are derived from *Drosophila melanogaster*. These cells were also transfected with DNA plasmids coding for the A^{d} α-chain containing a biotinylation sequence and the A^{g7} β-chain N-terminally connected to the 2.5mi peptide or the $GPI_{282-292}$ peptide. Purified molecules were biotinylated with BirA enzyme (Avidity, Aurora, CO, USA).

A^{g7}/2.5mi peptide MHC class II tetramers were generated incubating 5 µM A^{g7} monomers with 1 µM PE-labelled streptavidin (Sigma) in a ratio 5:1 at 37 °C for 1 hour in the dark. A^{g7}/GPI peptide MHC class II tetramers were generated in the same manner as irrelevant-peptide controls. Simultaneously, single-cell suspensions were obtained by mechanical disruption of the spleen, PLN and MDLN obtained from NOD mice 4 days after PS2.5mi-liposomes treatment. Cell suspensions obtained from spleens were lysed with haemolysis solution [500 mL deionised H_2O (Milli-Q® Direct, Merck Millipore, Burlington, MA, USA) plus 1.0297 g Trizma Hydrochloride (Sigma) and 3.735 g NH_4Cl (Probus, Badalona, Spain)] prior to cell staining. Firstly, endogenous biotin in the cell suspensions was blocked with 30 µL of 500 µg/mL avidin (Sigma) for 1 hour at room temperature. Then, cells were washed at 9,300 × g for 30 seconds and suspended with 0.1 µM PE-labelled A^{g7}/2.5mi peptide MHC class II tetramers to be incubated for 30 minutes at 4 °C. Then, the antibodies CD4 PB, CD8 PECy5, CD19 PECy5, F4/80 PEcy5, CD11c PECy5 (BioLegend, San Diego, CA, USA), CD49b FITC and LAG3 APC (BD Biosciences, San Jose, CA, USA) were added to the mix and incubated at 37 °C for 30 minutes. Further information on the antibodies used can be found in **Table 5**. Finally, cells were washed in PBS (Oxoid Limited, Hampshire, UK) and stained with 7-Amino-

Table 5. Panel of fluorophore-labelled monoclonal antibodies employed in the study of MHC class II tetramer-stained antigen-specific T cells

Target	Fluorophore	Species/Isotype	Clone	Use (µg/mL)	Company
CD4	PB	Rat IgG2a, κ	RM4-5	2.5	BioLegend
CD8	PECy5	Rat IgG2a, κ	53-6.7	1	BioLegend
CD11c	PECy5	Armenian Hamster IgG	N418	1	BioLegend
CD19	PECy5	Rat IgG2a, κ	6D5	1	BioLegend
CD49b	FITC	Rat (LEW) IgM, κ	DX5	5	BD Biosciences
LAG3	APC	Rat (LEW) IgG1, κ	C9B7W	2	BD Biosciences
F4/80	PECy5	Rat IgG2a, κ	BM8	1	BioLegend

PB: pacific blue; PECy5: phycoerythrin-cyanine 5; FITC: fluorescein isothiocyanate; APC: allophycocyanin.

Actinomycin D (7-AAD, BD Biosciences) in order to exclude dead cells upon cytometer analysis. Flow cytometry was carried out using FACSCanto II (BD Biosciences), acquiring at least 200,000 $CD4^+$ events. Fluorescence minus one (FMO) controls were used to determine positivity of CD49b and LAG3 staining. Data were analysed using FlowJo software (Tree Star, Ashland, OR, USA).

4.2. Detection of antigen-specific $CD8^+$ T cells in NOD mice after PS-liposomes treatment

To study changes in $CD8^+$ T cell subsets, $H2K^d$/NRP-V7 peptide MHC class I tetramers were employed. NRP-V7 peptide (N-start-KYNKANWFL-C-end, **Table 4**) is a mimotope agonist peptide for the diabetes-accelerating $CD8^+$ T cell clone named NY8.3, which is reactive against IGRP (Lieberman *et al.*, 2003). In the same manner as the A^{g7}/2.5mi tetramers do, $H2K^d$/NRP-V7 tetramers recognise NRP^+ T lymphocytes. Thus, in order to detect NRP^+CD8^+ T cells with $H2K^d$/NRP-V7 tetramers, female prediabetic 8-14 week old NOD mice were administered i.p. with a single 3.5 mg lipid of PSNRP-liposomes dose in 200 µL of PBS (Hyclone, GE Healthcare Life Sciences). Control groups received either 200 µL of PCNRP-liposomes, one injection of NRP-V7 peptide diluted in 200 µL PBS (Hyclone, GE Healthcare Life Sciences) in accordance with PSNRP-liposomes encapsulation efficiency, or saline solution (PBS, sham). Mice were also monitored for glycosuria and euthanised by cervical dislocation 4 days after the injection to harvest spleen, PLN and MDLN.

Both $H2K^d$/NRP-V7 peptide (N-start-KYNKANWFL-C-end, **Table 4**) and $H2K^d$/TUM peptide (N-start-KYQAVTTTL-C-end) MHC class I monomers were generously lent by Dr John Priatel (Child and Family Research Institute, British Columbia's Children's Hospital). Briefly, both $H2K^d$ molecule and mouse β2-microglobulin were synthesised in *Escherichia coli* and refolded with the proper peptide *in vitro*, as described (Vincent *et al.*, 2010). Peptide-MHC monomers were purified by high-performance liquid chromatography (HPLC) before biotinylation with biotin protein ligase.

$H2K^d$/NRP-V7 peptide MHC class I monomers at 0.57 µg/µL were incubated for 105 minutes at room temperature with 0.18 µg/µL PE-labelled streptavidin (Sigma) in a ratio 3.2:1 ratio to assemble $H2K^d$/NRP-V7 peptide MHC class I tetramers, diluted in a final volume of 50 µL with PBS (Oxoid Limited) with 2% foetal bovine serum (FBS, ThermoFisher Scientific) media. $H2K^d$/TUM peptide MHC class I tetramers were generated as irrelevant-peptide controls. At the same time, cell suspensions were obtained as described in section 4.1. After discarding supernatant (9,300 × g for 30 seconds), 50 µL of mounted tetramer were added directly to cell pellet and suspended, prior to incubation for 30 minutes at 4 °C. Afterwards, 10 µL fluorophore-labelled monoclonal antibodies CD8 FITC, CD19 V450 and Gr-1 eF660 (de-

tailed in **Table 6**) were added and incubated for 30 minutes at 4 °C. Cells were washed with 1 mL PBS (Oxoid Limited) with 2% FBS (ThermoFisher Scientific) media and suspended with 1 µL 7-AAD (BD Biosciences) in 49 µL PBS (Oxoid Limited) to proceed to cytometer analysis. This was performed using FACSCanto II (BD Biosciences), acquiring at least 200,000 $CD8^+$ events. Data were analysed using FlowJo software (Tree Star).

Table 6. Panel of fluorophore-labelled monoclonal antibodies employed in the study of MHC class I tetramer-stained antigen-specific T cells

Target	Fluorophore	Species/Isotype	Clone	Use (µg/mL)	Company
CD8	FITC	Rat (LOU) IgG2a, κ	53-6.7	1	BD Biosciences
CD19	V450	Rat (LEW) IgG2a, κ	1D3	1	BD Biosciences
Gr-1	eF660	Rat IgG2b, κ	RB6-8C5	2	ThermoFisher Scientific

FITC: fluorescein isothiocyanate; V450: violet 450; eF660: eFluor 660.

4.3. Evaluation of toxicity and tolerability of PS-liposomes treatment

In order to determine the tolerability and toxicity of the final PS-liposomes product, severe administration patterns and posology were tested in NOD mice. In the first assay, 6 prediabetic (8-12 week old, 3 male and 3 female) NOD mice were injected i.p. daily with a 200 µL dose of 3.5 mg (30 mM) PS-liposomes (PS30-liposomes) for 14 consecutive days. PBS (Hyclone, GE Healthcare Life Sciences) i.p. administration was used as control (sham) in 6 additional mice (3 male and 3 female). The study lasted for 21 days, during which the welfare of animals was strictly monitored in terms of weight, glucosuria, physical appearance (fur, presence of secretions or bruises) and behaviour (barbering, physical activity, aggressiveness, stereotyped activities and pain), as shown in **Table 7**. Mice were euthanised in the case that the total score reached a maximum value of 3 or at the end of the follow-up period. In order to obtain sera, 4 mice per group were euthanised to collect 500 µL of blood by cardiac puncture and necropsied. Blood samples were centrifuged at 600 × g for 5 minutes, followed by a second centrifugation at 2,300 × g for 5 minutes, and the sera were analysed for different biochemical parameters, such as total CH, high-density lipoprotein (HDL) CH, triglycerides, creatinine, urea and alanine aminotransferase (ALT) concentrations. In the second assay, 3 prediabetic NOD mice (2 female and 1 male) were injected i.p. with only one 200 µL dose of 10.5 mg (90 mM) PS-liposomes (PS90-liposomes) and euthanised 7 days afterwards; 3 additional mice (1 female and 2 male) were injected with PBS i.p. as control. The physical and behavioural status of the mice was monitored as described for the first assay (**Table 7**).

Table 7. Criteria used in welfare monitoring of the mice employed for the toxicity and tolerability assays of the PS-liposomes final product

Parameters			Score
Animal body weight	Usual body weight (animal is not underweighted and grows normally)		0
	Loss of 10% of body weight		1
	Loss of 10-20% of body weight		2
	Loss of ≥20% of body weight		3
Animal glucosuria	Absence of glucosuria		0
	Presence of glucosuria		1
Animal physical monitoring	Appearance	Good overall state	0
		Bad condition of fur	1
		Abnormal posture	1
		Piloerection	2
		Cachexia	3
	Secretions	Absence of ocular or nasal secretions	0
		Presence of ocular secretions	1
		Presence of nasal secretions	1
	Bruises	Absence of bruises	0
		Mild bruises on the ears	1
		Severe bruises on the ears	2
		Mild bruises on the abdomen	1
		Severe bruises on the abdomen	2
		Mild bruises on the hind limbs	1
		Severe bruises on the hind limbs	2
		Mild bruises on the tail	1
		Severe bruises on the tail	2
		Auto-mutilation	3
Animal behavioural monitoring	Barbering	Mild or only affecting the snout	1
		Severe or corporal	2
	Physical activity	Normal or usual physical activity	0
		Reduced physical activity	1
		Reduced stimuli-driven response	1
	Aggressiveness	Mild aggressive behaviour	1
		Severe aggressive behaviour	2
	Stereotyped activities	Mild circling	2
		Severe or rapid circling	3
	Vocalisation indicating pain	No	0
		Yes	3
		Upon palpation	3

Adapted from *The severity assessment framework*, within the context of Directive 2010/63/EU on the protection of animals used for scientific purposes (ec.europa.eu/environment/chemicals/lab_animals/interpretation_en.htm).

4.4. Administration of PS-liposomes to prevent EAE development and to analyse changes in T cell subsets

After the induction of EAE to C57BL/6 female mice, the preventive potential of PSMOG-liposomes was tested. Thus, PSMOG-liposomes were twice injected i.p. at a dose of 1.75 mg lipid in 100 µL PBS (Hyclone, GE Healthcare Life Sciences), at days 5 and 9 post-EAE induction. In this manner, the effect of the immunotherapy was determined in a preclinical setting, at a time when autoimmune myelin destruction was ongoing but clinical signs had yet to manifest (occurring typically at day 13 post-induction). As controls, mice received either two injections of 100 µL empty PS-liposomes, two injections of dissolved MOG_{40-50} peptide in 100 µL PBS (Hyclone, GE Healthcare Life Sciences) in accordance with PSMOG-liposomes encapsulation efficiency, or two injections of saline solution (PBS, Hyclone, GE Healthcare Life Sciences) as sham. Mice were euthanised 15 days after immunisation, thus meaning 6 days after PSMOG-liposomes treatment, in order to identify the induced tolerogenic mechanism. Otherwise, mice —pooled from two independent experiments— were euthanised 30 days after PSMOG-liposomes administration in order to analyse its preventive effect against the disease.

Thus, changes in regulatory T cells were analysed in EAE-induced C57BL/6 mice 6 days after PSMOG-liposomes treatment. This checkpoint was chosen based on the time course of the EAE model, since the MOG-specific $CD4^+$ T lymphocyte response takes places 7 days after EAE induction. After obtaining the spleens from the mice, splenocyte suspensions were obtained by mechanical disruption and erythrocytes were further lysed with haemolysis solution (see section 4.1). Percentages of classical Treg cells were assessed by flow cytometry after membrane molecule staining with CD3 V450, CD4 APCCy7 and CD25 PE and intracellular staining with FoxP3 APC (ThermoFisher Scientific). Further information on antibodies used can be found in **Table 8**. To do so, cells were first stained with antibodies targeting membrane molecules at 4 °C for 20 minutes. Then, cells were washed in PBS at 400 × g for 5 minutes and 500 µL Fixation/Permeabilisation working solution (FoxP3 Transcription Factor Staining Buffer Kit, ThermoFisher Scientific) was added and incubated for 1 hour at 4 °C to fix and permeabilise splenocytes. After that, cells were washed with 1 mL 1X Permeabilisation buffer (400 × g, 5 minutes, at room temperature) and the supernatant was discarded. Fc receptors were blocked with 1.25 µL anti-mouse CD16/32 (ThermoFisher Scientific) in 50 µL Permeabilisation Buffer for 15 minutes at 4 °C prior to adding 1 µL anti-mouse FoxP3 APC in 50 µL Permeabilisation Buffer, which was incubated for 30 minutes at 4 °C. Finally, cells were washed in 1 mL Permeabilisation Buffer at 400 × g for 5 minutes and suspended in 150 µL Permeabilisation Buffer before acquisition with FACSCanto II (BD Biosciences). Samples

were gated using forward scatter (FSC) and side scatter (SSC) to exclude dead cells and debris. FMO controls were used to assess CD25 and FoxP3 staining positivity. Data were analysed using FlowJo software (Tree Star).

Percentages of non-classical Treg cells, TR1 cells (Gagliani *et al.*, 2013), among splenocytes were assessed with CD3 V450, CD4 APCCy7 (ThermoFisher Scientific), CD49b FITC and LAG3 APC (BD Biosciences) staining, incubated at 4 °C for 20 minutes. Detailed information on antibodies used is shown in **Table 8**. Splenocytes were further washed in PBS (Oxoid Limited) at 400 × g for 5 minutes at room temperature and stained with 7-AAD (BD Biosciences) in order to exclude dead cells. Data were analysed using FlowJo software (Tree Star).

Table 8. Panel of monoclonal antibodies used in the study of T cell phenotype after PSMOG-liposomes treatment

Target	Fluorophore	Species/Isotype	Clone	Use (µg/mL)	Company
CD3	V450	Syrian hamster IgG	500A2	2	ThermoFisher Scientific
CD4	APCCy7	Rat IgG2b, κ	GK1.5	2	ThermoFisher Scientific
CD16/32	Purified	Rat IgG2a, λ	93	12.5	ThermoFisher Scientific
CD25	PE	Rat IgG1, λ	PC61.5	2	ThermoFisher Scientific
FoxP3	APC	Rat IgG2a, κ	FJK-16s	4	ThermoFisher Scientific
CD49b	FITC	Rat (LEW) IgM, κ	DX5	5	BD Biosciences
LAG3	APC	Rat (LEW) IgG1, κ	C9B7W	2	BD Biosciences

V450: violet 450; APCCy7: allophycocyanin-cyanine 7; PE: phycoerythrin; APC: allophycocyanin, FITC: fluorescein isothiocyanate.

5. Evaluation of the effect of PS-liposomes in DCs *in vitro*

As prominent APCs, DCs bridge the innate and adaptive responses and orchestrate the immune response. To investigate whether the engulfment of PS-liposomes by DCs tipped the scale towards immunogenicity or tolerance, different parameters were assessed employing flow cytometry, soluble molecule measurement and genomic analysis.

5.1. Cell viability

The viability of DCs cultured with PS-liposomes was assessed with Annexin V PE (Immunotools, Friesoythe, Germany) and 7-AAD (BD Biosciences) staining, which

were incubated for 15 minutes at room temperature. Samples were analysed using flow cytometry (FACS Canto II, BD Biosciences).

5.2. Generation of murine DCs from bone marrow precursor cells

DCs were differentiated from bone marrow precursor cells obtained from 8-14 week old female EAE-induced C57/BL6 mice at day 7 post induction. Briefly, mice were euthanised and hind leg bones (femur and tibia) were isolated after carefully removing the adjacent muscles and residue tissues. Both ends of the bones were cut off and bone marrow was flushed out with complete RPMI media, which contained RPMI-1640 culture medium (Biowest, Nuaillé, France) supplemented with 10% heat-inactivated FBS (ThermoFisher Scientific), 100 IU/mL penicillin (Normon SA, Madrid, Spain), 100 µg/mL streptomycin (Laboratorio Reig Jofré, Sant Joan Despí, Spain), 1 mM sodium pyruvate (ThermoFisher Scientific), 2 mM glutamine (Sigma), 50 µM β-mercaptoethanol (Sigma). Afterwards, erythrocytes were lysed with haemolysis solution (see section 4.1) for 5 minutes at room temperature. After washing, viability and count number of recovered cells were assessed with viability staining (see section 5.1) and Perfect Count Microspheres (Cytognos SL, Salamanca, Spain) using flow cytometry (FACS Canto II, BD Biosciences). Bone marrow precursor cells were cultured at 10^6 cells/mL in 24-well plates (Labclinics, Barcelona, Spain) suspended in complete RPMI media, produced as aforementioned, supplemented with mouse 1,000 IU/mL GM-CSF (Prospec, Rehovot, Israel) to obtain bone marrow-derived DCs. They were maintained in an incubator at 37 °C and 5% CO_2 for 8 days. Media and cytokine stimuli were replenished on alternate days. Viability, DC differentiation yield and number of cells were determined by viability staining (see section 5.1), CD11c PECy7 (clone HL3, BD Biosciences) staining and Perfect Count Microspheres (Cytognos SL) using flow cytometry (FACS Canto II, BD Biosciences).

5.3. Generation of human DCs from peripheral blood monocytes

Fifty-millilitre blood samples from adult subjects were collected in BD Vacutainer Sodium Heparin tubes (BD Biosciences). PBMCs were obtained by means of Ficoll Paque (GE Healthcare Life Sciences) density gradient centrifugation. Briefly, whole blood was diluted 1:1 with PBS (Oxoid Limited), and 2 volumes of diluted blood were added carefully along the wall of the tube to 1 volume of Ficoll Paque. Tubes were centrifuged at 754 × g without brake for 30 minutes at room temperature. The PBMCs layer was drawn off with a Pasteur pipette, and platelets were further removed by centrifugation at 200 × g for 15 minutes. The pelleted cells were suspended in PBS (Oxoid Limited) with 2% FBS (ThermoFisher Scientific) media, and viabil-

ity and number of cells were determined by viability staining (see section 5.1) and Perfect Count Microspheres (Cytognos SL) using flow cytometry (FACS Canto II, BD Biosciences). PBMCs were suspended to a concentration of 100×10^6 cells/mL and transferred to a Falcon 5 mL Polystyrene Round-Bottom Tube (BD Biosciences) to proceed to magnetic monocyte isolation using the EasySep Human CD14 Positive Selection Kit (STEMCELL Technologies, Vancouver, Canada). The tenth volume of CD14 Positive Selection cocktail was added to the cell suspension and incubated at room temperature for 15 minutes; afterwards, the twentieth volume of Magnetic Nanoparticles was added and incubated for 10 minutes at room temperature. The cell suspension was brought up to 2.5 mL with PBS (Oxoid Limited) with 2% FBS (ThermoFisher Scientific) media and the tube was then placed into the EasySep magnet (STEMCELL Technologies). The supernatant fraction was poured off after 5 minutes of incubation, and remaining cells were mixed with PBS (Oxoid Limited) with 2% FBS (ThermoFisher Scientific) media. Magnet incubations were repeated until CD14 purity in the positively selected fraction was >70%, which was checked by CD14 PE (Immunotools) staining altogether with 7-AAD (BD Biosciences) and number of cells as aforementioned. Monocytes were cultured in 24-well plates (Labclinics) at a concentration of 10^6 cells/mL in complete X-VIVO 15 media (Lonza, Basel, Switzerland), supplemented with 2% male AB human serum (Biowest), 100 IU/mL penicillin (Normon SA), 100 µg/mL streptomycin (Laboratorio Reig Jofré), and 1,000 IU/mL IL-4 and 1,000 IU/mL GM-CSF (Prospec) to obtain monocyte-derived DCs. They were maintained in an incubator at 37 °C and 5% CO_2 for 6 days. Media and cytokine stimuli were replenished on day 4. Viability, purity and number of differentiated DCs were assessed with viability staining (see section 5.1), CD11c APC (clone BU15, Immunotools) staining and Perfect Count Microspheres (Cytognos SL) using flow cytometry (FACS Canto II, BD Biosciences). Viability and number of cells in the negatively selected fraction of PBMCs were also determined by flow cytometry (FACS Canto II, BD Biosciences), and the cells were cryopreserved in FBS (ThermoFisher Scientific) with 10% DMSO (Sigma) at a concentration of $15\text{-}20 \times 10^6$ cells/mL and stored for later use.

Regarding paediatric 10 mL blood samples, they were collected in BD Vacutainer Sodium Heparin tubes (BD Biosciences) and monocytes were directly isolated from whole blood samples. Per 1 mL of blood, 50 µL of StraightFrom Whole Blood CD14 MicroBeads kit (Miltenyi Biotech, Bergisch Gladbach, Germany) were added and incubated for 15 minutes at 2-8 °C. A Whole Blood Column (Miltenyi Biotech) was placed in a MACS Separator (Miltenyi Biotech) and rinsed with 3 mL of MACS Buffer [PBS (Oxoid Limited) with 0.5% Human Albumin (Grifols, Sant Cugat del Vallès, Spain)], onto which the magnetically-labelled cell suspension was applied. The column was washed with MACS Buffer and then removed from the separator, and cells

were flushed out with 4 mL Whole Blood Column Elution Buffer (Miltenyi Biotech) by pushing a plunger into the column. Remaining erythrocytes were lysed with 5 mL haemolysis solution (see section 4.1). Viability and cell number of the $CD14^+$ positively-selected fraction were determined as abovementioned, and monocytes were derived into DCs in the same conditions as the ones obtained from adult subjects. On days 4 and 6, viability, CD11c percentage and number of differentiated DCs were assessed in the same manner as with adult DCs.

5.4. PS-liposomes capture kinetics by DCs

To determine PS-liposomes phagocytosis kinetics by DCs (either murine or human), they were cultured with 100 µM empty fluorescent PSOG488-liposomes at 37 °C from 5 minutes to 6 hours (for murine cells) or to 24 hours (for human cells). The same assay was performed at 4 °C to confirm that liposomes were captured by an active mechanism of phagocytosis. The phagocytosis kinetics PCOG488-liposomes was also determined in human DCs. After incubation, DCs were washed with 1 mL cold PBS (Oxoid Limited), vortexed for 15 seconds and centrifuged at 400 × g during 5 minutes at 4 °C to remove all liposomes attached to the cell membrane. This washing step was repeated 5 times. Fluorescent PSOG488-liposomes phagocytosis was assessed by flow cytometry (FACSCanto II, BD Biosciences) alongside viability staining (see section 5.1) and CD11c APC (clone N418, ThermoFisher Scientific) for murine cells, or CD11c APC (clone BU15, Immunotools) for human cells, incubated at 4 °C for 15 minutes.

5.5. DCs phenotype after PS-liposomes phagocytosis

DCs were differentiated from bone marrow precursor cells from EAE-induced mice in culture throughout 8 days. On day 7, 1 mM PSMOG-liposomes were added to the culture in order to assess their effect in DCs 24 hours afterwards (PSMOG-DCs). As controls, DCs were cultured either in basal conditions (immature DCs, iDCs), with 1 mM empty PS-liposomes (PS-DCs), with the equivalent amount of $MOG_{40\text{-}55}$ peptide (MOG-DCs) according to PSMOG-liposomes encapsulation efficiency, or with 100 ng/mL lipopolysaccharide (LPS, Sigma) to obtain mature DCs (mDCs). On day 8 of culture, supernatants of each well were stored at –20 °C for further analysis and DCs were detached from culture wells by exposure to 500 µL Accutase (ThermoFisher Scientific) during 20 minutes at 37 °C. Then, the cells were washed at 400 × g for 5 minutes, and the supernatant was decanted. DCs were stained with viability staining (see section 5.1) to analyse by flow cytometry (FACS Canto II, BD Biosciences). Afterwards, cells were stained with monoclonal antibodies to CD11c PECy7, CD40

APC, CD86 PE and MHC class II (I-A/I-E) FITC (BD Biosciences), for 20 minutes at 4 °C. Further information on the antibodies used can be found in **Table 9**. DCs were then washed in 1 mL PBS (Oxoid Limited) at 400 × g for 5 minutes at room temperature and suspended in 100 µL RPMI-1640 complete media (see section 5.2). Corresponding FMO staining was used as a control. Data were analysed using FlowJo software (Tree Star).

Table 9. List of monoclonal antibodies used for determining murine DCs phenotype

Target	Fluorophore	Species/Isotype	Clone	Use (µg/mL)	Company
CD11c	PECy7	Armenian Hamster IgG1, λ	HL3	2	BD Biosciences
CD40	APC	Rat IgG2a, κ	3/23	2	BD Biosciences
CD86	PE	Rat IgG2a, κ	GL1	0.2	ThermoFisher Scientific
MHC class II (I-A/I-E)	FITC	11-5321-81	M5/114.15.2	5	ThermoFisher Scientific

PECy7: phycoerythrin-cyanine 7; APC: allophycocyanin; PE: phycoerythrin; FITC: fluorescein isothiocyanate.

In the case of human samples, DCs were differentiated from peripheral blood in culture throughout 6 days. On day 5, 0.5 mM PSA-liposomes and 0.5 mM PSB-liposomes (PSAB-liposomes) were added to the DCs culture in order to assess the effect of the whole insulin molecule as autoantigen 24 hours afterwards (PSAB-DCs). As controls, DCs were cultured in basal conditions to obtain iDCs or adding a cytokine cocktail (CC) consisting of 1000 IU/mL TNF-α (Immunotools), 2000 IU/mL IL-1β (Immunotools) and 1 µM PGE_2, (Cayman Chemical, Ann Arbor, MI, USA) for 24 hours to obtain mDCs. Moreover, PSAB-DCs were cultured after phagocytosis with CC for 24 hours in order to assess their response in front of a pro-inflammatory stimulus (mPSAB-DCs). On day 6 of culture, supernatants were stored and frozen at –20 °C for further analysis. DCs were harvested from culture wells with 500 µL Accutase (ThermoFisher Scientific) as aforementioned. Afterwards, cells were equally washed. Cell viability was determined with viability staining (see section 5.1) by flow cytometry (FACS Canto II, BD Biosciences). Then, cells were stained with monoclonal antibodies CD11c APC, CD25 PE, CD86 FITC, HLA class I FITC, HLA class II FITC, CD14 PE and CD40 APC (Immunotools), CD36 APCCy7, TIM-4 APC, αvβ5 integrin PE, CD54 PECy7, TLR2 FITC, CXCR4 APCCy7, CCR2 APC, DC-SIGN APC (BioLegend) and CCR7 PECy7 (BD Biosciences). Further information on the antibodies used can be found in **Table 10**. DCs were stained for 20 minutes at 4 °C, then washed in 1 mL PBS (Oxoid Limited) at 400 × g for 5 minutes at room temperature and suspended in 100 µL X-VIVO 15 complete media (see section 5.3). Corresponding FMO staining was used as a control. Data were analysed using FlowJo software (Tree Star).

Table 10. List of monoclonal antibodies used for determining human DCs phenotype

Target	Fluorophore	Species/Isotype	Clone	Use	Company
CCR2	APC	Mouse IgG_{2a}, κ	K036C2	0.32 µg/mL	BioLegend
CCR7	PECy7	Rat IgG_{2a}, κ	3D12	1:50 dilution*	BD Biosciences
CD11c	APC	Mouse IgG_1	BU15	1:250 dilution*	Immunotools
CD14	PE	Mouse IgG_1	18D11	1:500 dilution*	Immunotools
CD25	PE	Mouse IgG_1	HI25a	1:100 dilution*	Immunotools
CD36	APCCy7	Mouse IgG_{2a}, κ	5-271	0.8 µg/mL	BioLegend
CD40	APC	Mouse IgG_1	HI40a	1:200 dilution*	Immunotools
CD54	PECy7	Mouse IgG_1, κ	HA58	0.4 µg/mL	BioLegend
CD86	FITC	Mouse IgG_1	BU63	1:100 dilution*	Immunotools
CXCR4	APCCy7	Mouse IgG_{2a}, κ	12G5	1.2 µg/mL	BioLegend
DC-SIGN	APC	Mouse IgG_{2a}, κ	9E9A8	4 µg/mL	BioLegend
HLA-ABC	FITC	Mouse IgG_{2a}	W6/32	1:100 dilution*	Immunotools
HLA-DR	FITC	Mouse IgG_1, κ	MEM-12	1:100 dilution*	Immunotools
Integrin αvβ5	PE	Mouse IgG_1, κ	P1F6	0.2 µg/mL	BioLegend
TIM-4	APC	Mouse IgG_1, κ	9F4	0.4 µg/mL	BioLegend
TLR2	FITC	Mouse IgG_{2a}, κ	TL2.1	1.6 µg/mL	BioLegend

*The company did not provide the stock concentration of the antibody. Instead, after prior titration, use dilution of the antibody is specified.

PB: pacific blue; FITC: fluorescein isothiocyanate; PECy5: phycoerythrin-cyanine 5; V450: violet 450; APC: allophycocyanin; eF660: eFluor 660.

5.6. DCs ability to stimulate autologous T lymphocyte proliferation

In order to determine human DCs ability to induce autologous T cell proliferation after phagocytosis of PS-liposomes, DCs were cocultured with autologous PBMCs. On day 5 of the differentiation protocol, all DCs conditions were loaded with 20 µg/mL human insulin (Sigma). DCs were then either cultured in basal conditions to obtain iDCs or with 0.5 mM PSA-liposomes and 0.5 mM PSB-liposomes (PSAB-DCs and mPSAB-DCs). The mPSAB-DCs and mDCs conditions were also exposed to CC (see section 5.5) two hours after receiving the aforementioned stimuli. Cells were maintained in the incubator at 37 °C and 5% CO_2 for 24 hours prior to coculture with autologous PBMCs.

Also, on day 5 of DC culture, autologous PBMCs were thawed in pre-warmed R-10 media [RPMI-1640 media (Biowest) plus 10% FBS (ThermoFisher Scientific)] and washed twice in PBS (Oxoid Limited) at 400 × g for 5 minutes at room temperature. Viability and cell number were determined with viability staining (see section 5.1) and Perfect Count Microspheres (Cytognos SL) using flow cytometry (FACS Canto II, BD Biosciences). Then, 16×10^6 cells were diluted in PBS (Oxoid Limited) at a final concentration of 2×10^6 cells/mL and stained with CellTrace Violet (CTV,

ThermoFisher Scientific). The CTV dye labels cells without affecting their viability or function and is used to assay cell proliferation over multiple generations by flow cytometry. This stain crosses the plasma membrane and covalently binds to all free amines located on the surface and inside of cells, to be distributed evenly between daughter cells upon division. Moreover, studying proliferation by flow cytometry allows for the use of other fluorophore-labelled monoclonal antibodies to determine which subsets are most affected by tolDCs. Briefly, 1 µL 5 mM CTV stock solution was diluted to 0.62 µM in 8,000 µL PBS (Oxoid Limited) and then mixed with the cell suspension to 1:1 volume, achieving a final working concentration of 0.31 µM CTV. This was incubated for 20 minutes at 37 °C in a pre-warmed bath; afterwards, any unbound dye was quenched with R-10 medium. Cells were centrifuged at 400 × g for 5 minutes at room temperature and suspended in 5 mL R-10 media, to be later incubated at 37 °C for at least 10 minutes to allow ester hydrolysis. PBMCs were washed in PBS (Oxoid Limited) at 400 × g for 5 minutes at room temperature and suspended in X-VIVO 15 complete media (see section 5.3) prior to assessing viability and cell number by flow cytometry (FACS Canto II, BD Biosciences) as abovementioned. PBMCs were harvested at a final concentration of 10^6 cells/mL and 100 µL (100,000 cells) were plated in 96-well round bottom plates (Labclinics).

On day 6 of DCs culture, supernatants of each DCs condition were stored at –20 °C for further analysis and DCs were harvested from 24-well plates (Labclinics) by exposure to 500 µL Accutase (ThermoFisher Scientific) during 20 minutes at 37 °C. The supernatant was removed after cells were washed (400 × g for 5 minutes), and cells were suspended in 500 µL complete X-VIVO 15 media (see section 5.3). Viability and cell number were determined with viability staining (see section 5.1) and Perfect Count Microspheres (Cytognos SL) using flow cytometry (FACS Canto II, BD Biosciences). DCs were then harvested at a final concentration of 0.1 × 10^6 cells/mL and 100 µL (10,000 cells) were cocultured with 100,000 autologous PMBCs (10:1 ratio, 10^5 PBMCs:10^4 DCs). For each donor, 10^5 PBMCs were cultured in triplicates in basal conditions as a negative control or with 50 ng/mL Phorbol 12-Myristate 13-Acetate (PMA, Sigma) and 500 ng/mL Ionomycin (Sigma) as a positive control.

After 6 days of coculture in the incubator at 37 °C and 5% CO_2, proliferation was assessed in the different T cell subsets with monoclonal antibodies to CD3, CD4 and CD8 molecules. Thus, supernatants of each well were collected and stored at –20 °C for further analysis. Cells were then washed in 150 µL PBS (Oxoid Limited) per well (400 × g, 5 minutes, room temperature); the supernatant was removed and staining mix containing 7-AAD (BD Biosciences), CD3 PE, CD4 APC and CD8 FITC (Immunotools) was incubated for 20 minutes at 4 °C. See **Table 11** for detailed

information on the antibodies used. After that, cells were washed in PBS (Oxoid Limited) as abovementioned and suspended in 200 µL PBS (Oxoid Limited) per well. Samples were acquired with FACS LSR Fortessa (BD Biosciences). Data were analysed using FlowJo software (Tree Star).

Table 11. List of monoclonal antibodies used in autologous T cell proliferation assay

Target	Fluorophore	Species/Isotype	Clone	Use	Company
CD3	PE	Mouse monoclonal IgG_1, κ	UCHT-1	1:100 dilution*	Immunotools
CD4	APC	Mouse IgG_{2a}	EDU-2	1:100 dilution*	Immunotools
CD8	FITC	Mouse IgG_1, κ	HIT8a	1:100 dilution*	Immunotools

*The company did not provide the stock concentration of the antibody. Instead, after prior titration, use dilution of the antibody is specified.

PE: phycoerythrin; APC: allophycocyanin; FITC: fluorescein isothiocyanate.

5.7. DCs secretion of soluble molecules

Aside from cell to cell interaction, the immune system makes use of several soluble molecules to modulate immune responses. Within this wide range of modulators, cytokines are low molecular weight (<30 kDa) proteins secreted by leukocytes and other cells that play a very prominent role in regulating the immune system. Additionally, other non-protein molecules, such as PGE_2, can also influence the outcome of immunoregulation.

5.7.1. Cytometric Bead Array

To assess the secretion of IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17A in supernatants of DCs culture and T cell proliferation assays, the Human Th1/Th2/Th17 Cytometric Bead Array (CBA) kit (BD Biosciences) was employed. This assay is based on seven capture bead populations, each coated with a cytokine-specific capture antibody, with distinct fluorescence intensities that are read in a red channel of a flow cytometer. These beads are mixed with the samples and incubated with PE-conjugated cytokine-detecting antibodies to form sandwich complexes, so that the intensity of PE fluorescence reveals the concentration of each cytokine, thus allowing for the measuring of multiple analytes simultaneously. Briefly, human cytokine standards were reconstituted (5,000 pg/mL) and diluted serially in two-fold steps to 1:256 dilution (20 pg/mL) and the seven capture beads were thoroughly vortexed and mixed. Then, an equal volume of pooled capture

beads, standards or samples and PE detection reagents were added to 96-well round bottom plates and incubated for 3 hours at room temperature. Afterwards, wash buffer (CBA system, BD Biosciences) was added to wash the wells (400 × g, 5 minutes and room temperature), the supernatant was discarded and 150 µL wash buffer was used to suspend the analytes within the wells. Samples were acquired with FACS LSR Fortessa (BD Biosciences). The theoretical limit of detection was 2.6 pg/mL for IL-2, 4.9 pg/mL for IL-4, 2.4 pg/mL for IL-6, 4.5 pg/mL for IL-10, 3.8 pg/mL for TNF-α, 3.7 pg/mL for IFN-γ and 18.9 pg/mL for IL-17A. Data were analysed using FCAP Array software (BD Biosciences).

5.7.2. *Enzyme-linked immunosorbent assay*

PGE_2 is an eicosanoid resulting from the metabolism of arachidonic acid, which is synthesised *de novo* upon cellular activation and released to the extracellular space. It possesses a prominent role in the induction of tolerance by DCs and is partly responsible for the immunosuppressive mechanism of apoptotic cells. Therefore, PGE_2 production by DCs 24 hours after PS-liposomes stimulus was assessed with the competitive immunoassay PGE_2 enzyme-linked immunosorbent assay (ELISA) kit (limit of detection: 15 pg/mL, sensitivity: 50 pg/mL; Cayman Chemical). Since lipid metabolism is highly conserved across vertebrates, the same assay can be used for mice and human samples. Being a competitive immunoassay, measurement of soluble PGE_2 present in the samples is based on the competition generated between this molecule and PGE_2-acetylcholinesterase conjugate (PGE_2 Tracer) for a limited amount of PGE_2 Monoclonal Antibody bound to the pre-coated plate. The protocol was performed as per manufacturer's instructions, and absorbance was read at a wavelength of 405 nm using a Varioskan microplate reader (ThermoFisher Scientific). Absorbance data were analysed with the spreadsheet made available by the company (Cayman Chemical).

TGF-β1 belongs to the TGF β superfamily of cytokines and is involved in various processes, ranging from proliferation, differentiation and migration of many cell types to immune function regulation. Thus, it is vastly expressed in different leukocyte cells, such as platelets, macrophages and lymphocytes. In this sense, TGF-β1 has been reported to possess an essential role in inducing self-tolerance after apoptotic cell clearance by phagocytes and in the development of Treg cells (Szondy *et al.*, 2017). TGF-β1 is synthesised alongside a Latency-associated peptide (LAP), which renders TGF-β1 inactive and must be cleaved for TGF-β1 to achieve its mature form. In this case, TGF-β1 production by human DCs after being exposed to PSAB-liposomes for 24 hours (see section 5.5) was measured using the TGF-β1 Human/Mouse Uncoated ELISA Kit (sensitivity 8 pg/mL; ThermoFisher Scientific)

on NUNC Maxisorp 96 well ELISA plates (ThermoFisher Scientific). The protocol was performed as instructed by the manufacturer. Absorbance was read at 450 nm using a Varioskan microplate reader (ThermoFisher Scientific) after 570 nm absorbance subtraction.

5.8. Gene expression profile through RNA-seq

To gain a deeper characterisation of the immunomodulatory effects exerted by PS-liposomes phagocytosis in DCs, a transcriptomic analysis employing RNA-seq was performed. To that end, 8 adult patients with T1D were selected with a more stringent inclusion and exclusion criteria (see section 3.2) in order to minimise the effect that long-term hyperglycaemia or glycaemia dysregulation over time could have on genetic and epigenetic profiles.

5.8.1. RNA isolation from DCs pellets

DCs obtained from these 8 patients were cultured in basal conditions (iDCs) or with 0.5 mM of PSA-liposomes and 0.5 mM of PSB-liposomes (PSAB-DCs) for 4 hours at 37 °C. Then, cells were harvested from culture wells after exposure to 500 µL Accutase for 20 minutes at 37 °C. Afterwards, cells were washed at 400 × g for 5 minutes, the supernatant was removed, and cells were suspended in 500 µL complete X-VIVO 15 media (see section 5.3). Viability, purity and number of DCs in each condition were assessed with viability staining (see section 5.1), CD11c APC (Immunotools) staining, and Perfect Count Microspheres (Cytognos SL) using flow cytometry (FACS Canto II, BD Biosciences). Next, cells were placed in 1.5 mL microcentrifuge tubes and centrifuged at 400 × g for 5 minutes to decant supernatant. Cells were suspended in 500 µL PBS (Oxoid Limited) and centrifuged again at maximum speed (16,000 × g) for 5 minutes. The supernatant was removed and cell pellets were stored at –80 °C until further use. Additionally, liposome capture control assays were performed for every sample (following the procedure described in section 5.4).

RNA was extracted from DC pellets using the RNeasy Micro kit (Qiagen, Hilden, Germany), which employs a silica-based membrane which selectively binds RNA to purify up to 45 µg RNA from small cell and tissue samples. This procedure allows for the purification of RNA molecules longer than 200 nucleotides, thus excluding shorter RNA molecules (5.8S rRNA, 5S rRNA, and tRNAs) and enriching for mRNA. The isolation was performed according to the manufacturer's instructions with few modifications. After thawing at 4 °C, cell pellets were immediately disrupted in 350 µL lysis buffer (RLT buffer) to inactivate RNases and prevent RNA degradation

and changes in the transcriptome. Next, lysates were homogenised by being subjected to vigorous vortex mixing, and the hundredth volume of β-mercaptoethanol (Sigma), 5 µL 4 ng/µL RNA carrier and one volume of 70% ethanol (Alcoholes Montplet, Barcelona, Spain) were added. The samples were pipetted thoroughly and transferred into an RNeasy MinElute spin column placed in a 2 mL collection tube to allow RNA material to bind to the silica membrane. Samples were washed with RW1 buffer (8,000 × g, 15 seconds) prior to and after incubation for 15 minutes with 80 µL 0.125X DNase I, added to eliminate the DNA material that might have bound to the silica membrane. Then, RPE buffer was added and washed (8,000 × g, 15 seconds) to discard flow-through. Next, 500 µL 80% ethanol were pipetted into the column and centrifuged twice at 8,000 × g for 2 minutes, followed by another centrifugation at maximum speed for 2 minutes with the lid of the columns opened. Finally, the RNeasy MinElute spin column was placed into a 1.5 mL collection tube and 14 µL of RNase-free water was added directly to the centre of the spin column membrane. RNA was eluted by centrifuging for 1 minute at full speed. RNA purity, integrity and concentration were determined using NanoDrop (ND-1000 Spectrophotometer, ThermoFisher Scientific) and 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA).

5.8.2. RNA library preparation, sequencing and data analysis

After isolation of RNA, 1 µg was used to prepare RNA libraries following the recommended protocol of the NebNext Ultra Directional RNA Library Prep Kit (New England Biolags, Ipswich, MA, USA). Library quality controls were assessed with a TapeStation 2200 (Agilent High Sensitivity Screen Tape) and, in all cases, a narrow distribution with a peak size of approximately 300 bp was observed. Then, libraries were quantified by qPCR using a QC KAPA kit (Hoffman-LaRoche, Basel, Switzerland) and sequenced in a NextSeq 500 genetic analyser (SBS-based sequencing technology, Illumina, San Diego, CA, USA) in a run of 2 × 75 cycles and a high output sequencing mode. Twenty million reads were obtained and analysed for each sample.

Fastq files obtained from the Illumina platform were merged prior to undergoing basic quality controls with FASTQC (Wingett and Andrews, 2018) and PRINSEQ tools (Schmieder and Edwards, 2011). On further processing, paired-end (forward-reverse) sample merging was performed with the software CLCBio Genomics Workbench® version 8.5 (Qiagen, 2017), as per instructed in CLCBio manuals. Read alignment and mapping steps were carried out using the same CLCBio software against the human genome (GRCh38 assembly, 2013) to both gene and transcript regions only, and default options were used to normalise counts by applying standard

"Reads Per Kilobase of transcript per Million reads mapped" (RPKM) method (Aanes *et al.*, 2014). The following steps of the analysis were carried out with scripts and pipelines implemented with R software (R Core Team, 2016). Normalised data were transformed to Log_2, and differentially expressed genes (DEGs) were selected with the linear model approach implemented in the limma Bioconductor package (Ritchie *et al.*, 2015). After adjusting the data for multiple testing with the False Discovery Rate method (Hochberg, 1995), adjusted *p*-values of ≤0.125 were considered for significance criteria. Therefore, genes with a *p*-value <0.0013 and Log_2 of fold change (FC) >0.05 were considered upregulated, whereas those with Log_2 of FC <–0.05 were considered downregulated. DEGs were classified into broad categories using Ingenuity Pathway Analysis software (Qiagen), Protein ANalysis THrough Evolutionary Relationships Classification System (PANTHER) (Mi *et al.*, 2013), REACTOME Pathway database (Fabregat *et al.*, 2016) and Gene Ontology Biological Process database (Blake *et al.*, 2015) in order to identify which signalling pathways could be considered as affected or unbalanced when comparing experimental conditions.

5.9. Gene expression profile through qRT-PCR

To validate the RNA-seq results, DCs were cultured and harvested as described in section 5.8.1. As an additional control, DCs were also cultured with CC for 4 hours at 37 °C to obtain mDCs. RNA was isolated from samples following the same procedure described in section 5.8.1.

After that, the synthesis of complementary DNA (cDNA) to the RNA extracted in the previous step was carried out by reverse transcription-polymerase chain reaction (RT-PCR). For all samples, a maximum of 1,000 ng of total RNA was reverse transcribed with a High Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific). cDNA synthesis was carried out mixing total RNA with 1 µL 0.5 mg/mL random primers (BioTools, Valle de la Tobalina, Spain) and adding RNase-free water to reach a total volume of 13.75 µL. Samples were then placed in a GeneAmp PCR System 9700 (ThermoFisher Scientific) to be incubated for 5 minutes at 70 °C. When cooled down, 1.25 µL 10 mM desxynucleosides (dNTPs, BioTools), 5 µL 5X transcriptase buffer (Promega, Madison, WI, USA), 0.5 µL 40 U/µL RNase Inhibitor (Promega) and 0.5 µL 200 U/µL reverse transcriptase Moloney Murine Leukaemia Virus (M-MLV, Promega) were added, along with 4 µL RNase-free water to reach a total volume of 25 µL. Samples were subjected to another cycle of incubation at 37 °C for 60 minutes.

If necessary (p.e. samples with less than 250 ng of total RNA), cDNA of genes of interest was pre-amplified prior to qPCR with TaqMan PreAmp Master Mix (ThermoFisher Scientific). Even though the same TaqMan Gene Expression Assays

of the genes of interest are used as a source for primers, this pre-amplification step does not introduce amplification bias to the sample. Thus, targeted cDNA pre-amplification was carried out mixing 25 µL 2X TaqMan PreAmp Master Mix, 12.5 µL 0.2X Pooled TaqMan Gene Expression Assays (ThermoFisher Scientific) and 12.5 µL retrotranscribed cDNA material. For the pre-amplification reaction, the following settings were used: 1 cycle to allow activation of the enzyme (10 minutes at 95 °C), 14 consecutive cycles to enable denaturation of DNA, annealing of the TaqMan probe to DNA and DNA extension (15 seconds at 95 °C followed by 4 minutes at 60 °C), 1 cycle to inactivate the enzyme (10 minutes at 99 °C) and 1 final cycle at 4 °C for the samples to cool down.

cDNA material was used as the starting material for the subsequent qPCR, performed on a LightCycler 480 (Roche, Mannheim, Germany) with TaqMan Gene Expression Assays (ThermoFisher Scientific). These assays contain unlabelled PCR primers and a TaqMan probe fluorescently-labelled on the 5' end and quenched on the 3' end so that, when annealing to its specific target sequence, the Taq polymerase can cleave the probe and separate the dye from the quencher. With each cycle of PCR, a greater number of dye molecules are released, resulting in an increase in fluorescence intensity that is proportional to the amount of amplicon synthesised. The TaqMan Assays used are listed in **Table 12**. Amplification of cDNA was carried out mixing 2 µL of cDNA sample, 0.75 µL 20X TaqMan Gene Expression Assay, 7.5 µL 2X TaqMan Universal Master Mix II with Uracil-N Glycosylase (UNG, ThermoFisher Scientific) and 4.75 µL RNase-free water to reach a final volume of 15 µL. On the other hand, in the case of preamplified samples, amplification of cDNA was carried out mixing 3.75 µL of 1:50-diluted preamplified cDNA product, 0.75 µL 20X TaqMan Gene Expression Assay, 7.5 µL 2X TaqMan Universal Master Mix II with UNG (ThermoFisher Scientific) and 3 µL RNase-free water to reach the final volume of 15 µL. For both pre-amplified and non-preamplified samples, each amplification

Table 12. List of TaqMan Assays used for analysing targeted gene expression in human DCs

Gene	Assay ID	Gene	Assay ID
CD36	Hs00354519_m1	*MERTK*	Hs01031979_m1
CD68	Hs00154355_m1	*MFGE8*	Hs00983890_m1
CYTH4	Hs01047905_m1	*PPARG*	Hs01115513_m1
GAPDH	Hs02758991_g1	*PLAUR*	Hs00958880_m1
GIMAP4	Hs01032964_m1	*TGFB1*	Hs00998133_m1
HPGD	Hs00960590_m1	*TNFAIP3*	Hs00234713_m1
NFKBIA	Hs00153283_m1	*TNFSF14*	Hs00542477_m1
LAIR1	Hs00253790_m1	*VEGFA*	Hs00900055_m1

was repeated in three replicates in 96-well PCR plates (96 Well Lightcycler plate, Sarstedt, Nümbrecht, Germany). After adding the reagents, plates were sealed with sealing tape (Sarstedt) and centrifuged for 1 minute at 1000 rpm. The amplification reaction had the following settings: first, 1 cycle to allow activation of UNG (2 minutes at 50 °C), 1 cycle to permit polymerase activation (10 minutes at 95 °C), 50 consecutive cycles to enable denaturation of DNA, annealing of the TaqMan probe to DNA and DNA extension (15 seconds at 95 °C followed by 1 minute at 60 °C) and, finally, 1 last cycle for 20 seconds at 40 °C to cool down the samples. Results were analysed by relative quantification method ($2^{-\Delta Cp}$) (Livak and Schmittgen, 2001), after normalising the expression of genes of interest to that of the housekeeping gene (*GAPDH*).

6. Analysis of DCs subsets in peripheral blood

In order to study the subsets of DCs present in peripheral blood, 2 mL blood samples were collected in ethylenediaminetetraacetic acid (EDTA) from paediatric control subjects and paediatric patients with T1D at the clinical onset and at 6, 12 and 18 months of disease progression. Whole blood samples were washed with PBS and erythrocytes were lysed with Lysing Buffer (BD Biosciences) as per manufacturer's instructions. A sample of 100 µL was then stained with CD45 AF700, CD3 APCH7, CD19 APCH7, CD14 V450, CD16 APC, CD11c PECy7, CD123 PerCPCy5.5, CD56 PE, HLA-DR V500 and SLAN FITC (BD Biosciences). Further information on the staining antibodies used can be found in **Table 13**. Samples were acquired with FACS LSR Fortessa (BD Biosciences), and a minimum of 10,000 leukocyte events

Table 13. List of monoclonal antibodies used for determining human peripheral blood DCs phenotype

Target	Fluorophore	Species/Isotype	Clone	Use	Company
CD3	APCH7	Mouse BALB/c IgG_1, κ	SK7	8.3 µg/mL	BD Biosciences
CD11c	PECy7	Mouse BALB/c IgG_1, κ	B-ly6	1:12 dilution*	BD Biosciences
CD14	V450	Mouse BALB/c IgG_{2b}, κ	MφP-9	1:12 dilution*	BD Biosciences
CD19	APCH7	Mouse IgG_1, κ	HIB19	1:12 dilution*	BD Biosciences
CD45	AF700	Mouse monoclonal IgG_1, κ	HI30	8.3 µg/mL	BD Biosciences
CD56	PE	Mouse BALB/c × C57BL/6 IgG1, κ	MY31	8.3 µg/mL	BD Biosciences
CD123	PerCPCy5.5	Mouse IgG_{2a}, κ	7G3	1:12 dilution*	BD Biosciences
HLA-DR	V500	Mouse IgG_{2a}, κ	G46-6	1:12 dilution*	BD Biosciences

*The company did not provide the stock concentration of the antibody. Instead, after prior titration, use dilution of the antibody is specified.

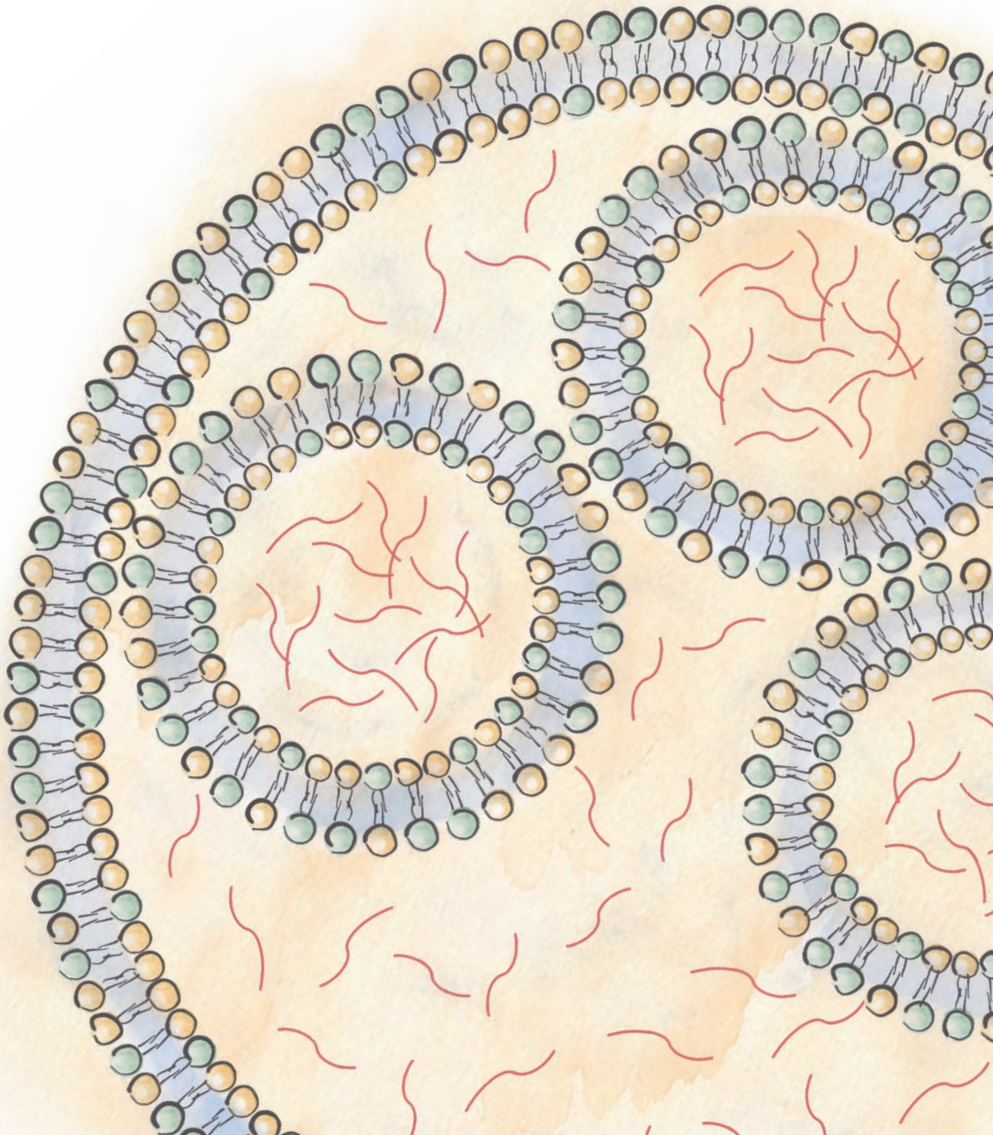
APCH7: allophycocyanin-hilite 7; PECy7: phycoerythrin-cyanine 7; V450: violet 450; AF700: alexa fluor 700; PE: phycoerythrin; PerCPCy5.5: peridinin-chlorophyll-cyanine 5.5; V500: violet 500.

per sample was acquired. Percentage and absolute counts (cells/μL) were analysed for all subsets using Perfect Count Microspheres (Cytognos SL). The gating strategy to analyse specific leukocyte subsets was based on international consensus (Robb *et al.*, 2016). Data were analysed using FACSDiva software (BD Biosciences).

7. Statistical analysis

Prism 7.0 software (GraphPad software Inc., San Diego, CA, USA) was used for the statistical analysis. For comparisons of unpaired data, a parametric *t* test or non-parametric Mann-Whitney test were used; for paired comparisons, a non-parametric Wilcoxon test was used. Analysis of variance (ANOVA) was used for comparisons with several factors. Fisher's exact test was employed to compare qualitative variables. For the correlation between parameters, the Spearman's test was used. A p-value ≤0.05 was considered significant.

RESULTS



SECTION I: PS-LIPOSOMES TREATMENT ELICITS THE EXPANSION OF ANTIGEN-SPECIFIC CD4$^+$ AND CD8$^+$ T CELLS AND DISPLAYS A SAFE PROFILE IN NOD MICE

The first part of this work aimed to dig deeper into the antigen-specific T cells expanded by autoantigen-loaded PS-liposomes treatment. We knew from previous results that this strategy expanded antigen-specific CD4$^+$ T cells, and that this expansion took place not only in the FoxP3$^+$ compartment but in the FoxP3$^-$ compartment as well (Pujol-Autonell *et al.*, 2015). FoxP3$^+$CD4$^+$ T cells are classical Treg cells that obviously contribute to arresting the autoimmune reaction in the NOD experimental model. Since these cells are only part of the picture, two new objectives were raised. The first was directed at finding whether PS-liposomes cause the expansion of antigen-specific CD4$^+$ non-classical TR1 cells that could also aid in the stopping of autoimmunity. To answer that, we analysed the intensity of CD49b and LAG3 markers in the expanded 2.5mi$^+$CD4$^+$ T cell subset using A^{g7}/2.5mi peptide MHC class II tetramers. The second objective targeted the other arm of the adaptive cellular response, the CD8$^+$ T cell compartment. Thus, we used H2K^d/NRP-V7 peptide MHC class I tetramers to detect the expansion of antigen-specific CD8$^+$ T cells upon PS-liposomes treatment in NOD mice. Furthermore, severe posology and administration patterns were tested in NOD mice to assess the safety and tolerability of the final PS-liposomes product.

1. PS-liposomes encapsulate T1D specific autoantigens

PS-liposomes were generated with PS/PC/CH at 1:1:1.33 molar ratio in order to present PS on their surface and simulate apoptotic cells, whereas PC-liposomes were produced as controls with PC/CH at 1:1 molar ratio. They were characterised in terms of lipid concentration, diameter size, PdI —meaning the distribution of sizes within the liposome sample—, surface charge (ζ-potential) and encapsulation efficiency of the respective peptide. All batches of liposomes had a final lipid concentration of 30 mM —except for PS90-liposomes, which were only used to test a severe dose administration— and a minimum of 500 nm diameter to guarantee efficient

phagocytosis. Depending on the presence or absence of PS in the composition, liposomes had a negative ζ-potential or a more neutral one, respectively. **Table 14** displays the features of the liposomes used in SECTION I. Of note, differences in 2.5mi peptide encapsulation efficiency in PS2.5mi-liposomes and PC2.5mi-liposomes can be explained by its net charge at pH 7 (0.2 mV, **Table 4**), which probably contributes to a higher encapsulation efficiency in more negatively-charged PS-rich liposomes.

Table 14. Physicochemical features of the liposomes used in SECTION I

Liposome nomenclature	Number of batches	Lipid concentration (mM)	Diameter size (nm)	PdI	ζ-potential (mV)	Encapsulation efficiency (%)
PS2.5mi-liposomes	1	29.96	506	0.19	–35.00	51.16
PC2.5mi-liposomes	1	29.96	913	0.18	–5.00	21.35
PSNRP-liposomes	2	30.04 ± 0.12	876 ± 140	1	–33.00 ± 2.97	20.11 ± 20.37
PCNRP-liposomes	2	30.01 ± 0.07	980 ± 33	0.62 ± 0.03	–2.87 ± 0.18	14.43 ± 1.99
PS30-liposomes	1	30.00	645	0.638	–35.9	—
PS90-liposomes	1	90.00	1,015	0.599	–31.3	—

Data presented as mean ± SD. PdI: polydispersity index.

2. Antigen-specific CD4$^+$ T cells expanded by PS-liposomes treatment express high levels of CD49b and LAG3 markers

Aiming to answer whether the 2.5mi$^+$CD4$^+$ T cells expanded by autoantigen-loaded PS-liposomes treatment could be TR1 cells, NOD mice were administered with PS2.5mi-liposomes and euthanised 4 days after to harvest the spleen, PLN and MDLN. As controls, mice received PBS (sham) or PC2.5mi-liposomes. Then, antigen-specific 2.5mi$^+$CD4$^+$ T cells and their CD49b and LAG3 expression —characteristic of the TR1 subset— were assessed with the use of A^{g7}/2.5mi peptide MHC class II tetramers (**Figure 8** and **Supplementary Figure 1**). First, we ensured that PS2.5mi-liposomes treatment induced an expansion of 2.5mi$^+$ CD4$^+$ T cells in NOD mice in these secondary lymphoid organs. In the spleen (**Figure 8A**), treatment with PS2.5mi-liposomes or PC2.5mi-liposomes caused a significant expansion of 2.5mi$^+$CD4$^+$ T cells when compared to sham treatment (p<0.01). No apparent differences were found between 2.5mi$^+$CD4$^+$ T cell expansions caused by PS2.5mi-liposomes and PC2.5mi-liposomes. In both PLN and MDLN (**Figure 8B-C**), admin-

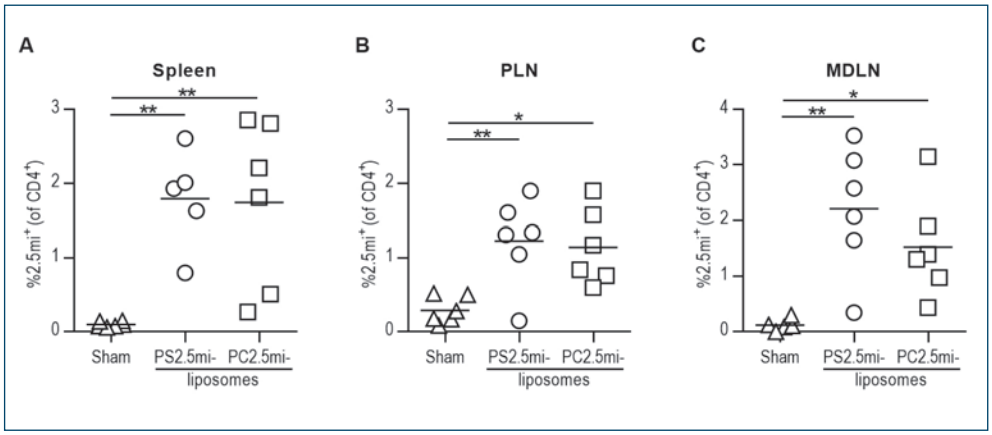


Figure 8. ***Antigen-specific CD4⁺ T cells are expanded 4 days after i.p. administration of PS-liposomes to NOD mice.*** **(A)** *Detection of 2.5mi⁺CD4⁺ T cells in the spleen,* **(B)** *PLN and* **(C)** *MDLN of NOD mice treated with PBS (n=6, sham, triangles), PS2.5mi-liposomes (n=5-6, circles), and PC2.5mi-liposomes (n=6, squares). Each symbol represents one animal and lines show the mean of all included mice, pooled from 6 independent experiments. Comparisons between groups showed significant differences (*p<0.05, **p<0.01, One-way ANOVA and Bonferroni multiple comparison test).*

istration of PS2.5mi-liposomes elicited a notable 2.5mi⁺CD4⁺ T cell expansion when compared to sham group (p<0.01), and treatment with PC2.5mi-liposomes likewise caused a significant expansion of these cells (p<0.05). Albeit no differences were found when comparing the clonal expansion caused by PS2.5mi-liposomes and PC2-5mi-liposomes in these two organs, the PS2.5mi-liposomes group showed a trend for a greater expansion in MDLN.

Once the expansion of 2.5mi⁺CD4⁺ T cells was confirmed, the level of expression of CD49b and LAG3 markers on these cells and their 2.5mi⁻CD4⁺ T cell counterparts was determined (**Figure 9**). This was measured through the assessment of median fluorescence intensity (MFI) of each marker. As for CD49b expression, 2.5mi⁺CD4⁺ T cells always showed greater expression of CD49b than 2.5mi⁻CD4⁺ T lymphocytes (p<0.05) regardless of the treatment received (PS2.5mi-liposomes or PC2.5mi-liposomes) or the organ assessed (spleen, PLN or MDLN), as shown in **Figure 9A-C**. Regarding the expression of LAG3 on those cells, the same trend was observed. Specifically, both in the spleen (**Figure 9D**) and PLN (**Figure 9E**), 2.5mi⁺CD4⁺ T cells tended towards a higher expression of LAG3 than their 2.5mi⁻CD4⁺ T cell counterparts with both liposomes' treatments, although statistical significance was not reached (p=0.0625). In MDLN (**Figure 9F**), PS2.5mi-liposomes administration showed a tendency to increase LAG3 expression in 2.5mi⁺CD4⁺ T cells when compared to 2.5mi⁻CD4⁺ T cells, whereas PC2.5mi-liposomes treatment did induce a higher expression of this marker in these cells (p<0.05).

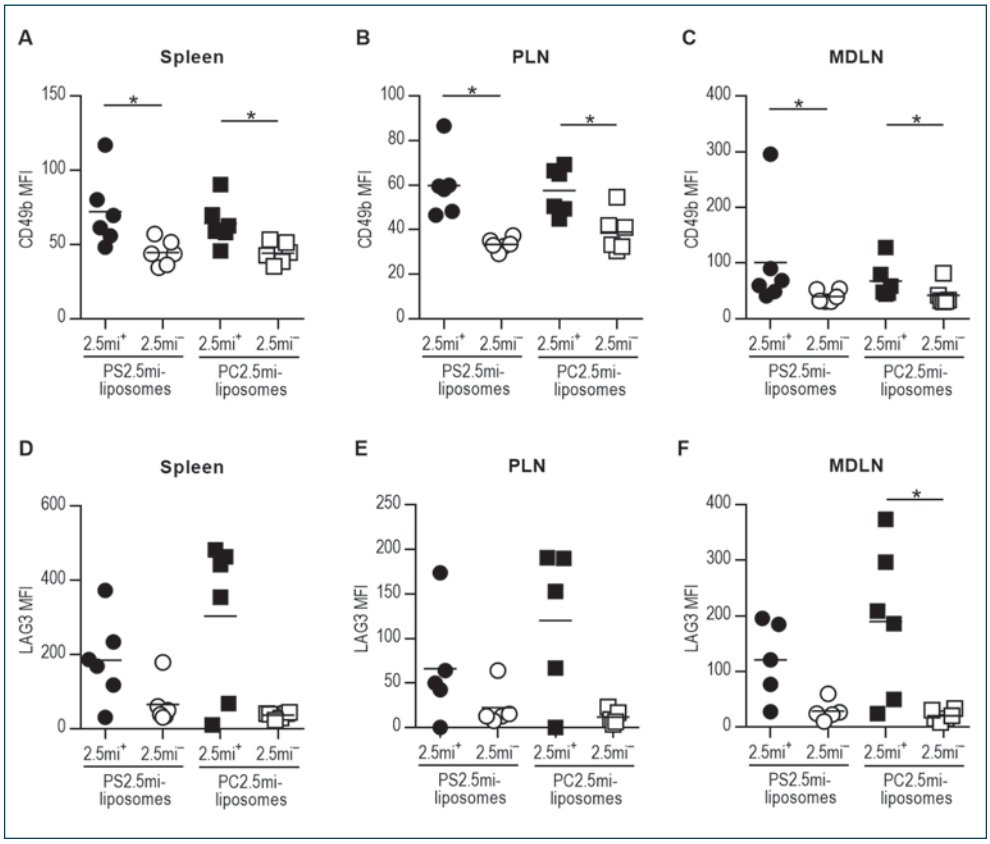


Figure 9. ***Antigen-specific CD4⁺ T cells express high levels of CD49b and LAG3 markers.*** **(A-C)** *MFI of CD49b surface marker in 2.5mi⁺CD4⁺ T cells (black symbols) and 2.5mi⁻CD4⁺ T cells (white symbols) in* **(A)** *spleen,* **(B)** *PLN and* **(C)** *MDLN from NOD mice treated with PS2.5mi-liposomes (n=6, circles) and PC2.5mi-liposomes (n=5-6, squares). Each symbol represents one animal and lines show the mean of all included mice, pooled from 6 independent experiments. Comparisons between paired groups showed statistically significant differences (*p<0.05, Wilcoxon test).* **(D-F)** *MFI of LAG3 surface marker in 2.5mi⁺CD4⁺ T cells (black symbols) and 2.5mi⁻CD4⁺ T cells (white symbols) in* **(D)** *spleen,* **(E)** *PLN and* **(F)** *MDLN from NOD mice treated with PS2.5mi-liposomes (n=5-6, circles) and PC2.5mi-liposomes (n=5-6, squares). Each symbol represents one animal and lines show the mean of all included mice, pooled from 6 independent experiments. Comparisons between paired groups showed statistically significant differences (*p<0.05, Wilcoxon test).*

3. Antigen-specific CD8⁺ T cells are expanded by PS-liposomes treatment

In order to detect antigen-specific CD8⁺ T cell expansion upon PS-liposomes treatment, NOD mice received one i.p. dose of PSNRP-liposomes. As controls, mice received PBS (sham), NRP-V7 peptide diluted in PBS or PCNRP-liposomes. Empty PS-liposomes or PC-liposomes were not considered as controls because they did

not elicit any antigen-specific CD4⁺ T cell expansion, as observed in previous experiments (Pujol-Autonell *et al.*, 2015). Mice were euthanised 4 days after treatment administration to collect the spleen, PLN and MDLN, and antigen-specific CD8⁺ T cell expansion was evaluated using H2K^d/NRP-V7 peptide MHC class I tetramers (**Figure 10** and **Supplementary Figure 2**). Thus, a higher percentage of NRP⁺CD8⁺ T lymphocytes was found in the spleen of PSNRP-liposomes-treated NOD mice when compared to mice treated with PBS (p<0.05, **Figure 10A**). Also, animals administered with PSNRP-liposomes showed a trend for a higher expansion of NRP⁺CD8⁺ T cells than those treated with NRP-V7 peptide (p=0.0625), but no differences were found when comparing the expansion caused by PSNRP-liposomes and PCNRP-liposomes. In PLN (**Figure 10B**), PSNRP-liposomes-treated mice exhibited the highest expansion of NRP⁺CD8⁺ T lymphocytes when compared to the other control conditions (p<0.05). In MDLN (**Figure 10C**), the highest expansion of NRP⁺CD8⁺ T lymphocytes was found in PSNRP-liposomes-treated mice in comparison to other groups (p<0.001 when compared to sham mice and mice treated with NRP-V7 peptide and p<0.01 when compared to mice treated with PCNRP-liposomes). It is worth noting that in both lymph nodes, the expansion of NRP⁺CD8⁺ T lymphocytes found in PSNRP-liposomes-treated mice was greater than the one exhibited by PCNRP-liposomes-treated mice.

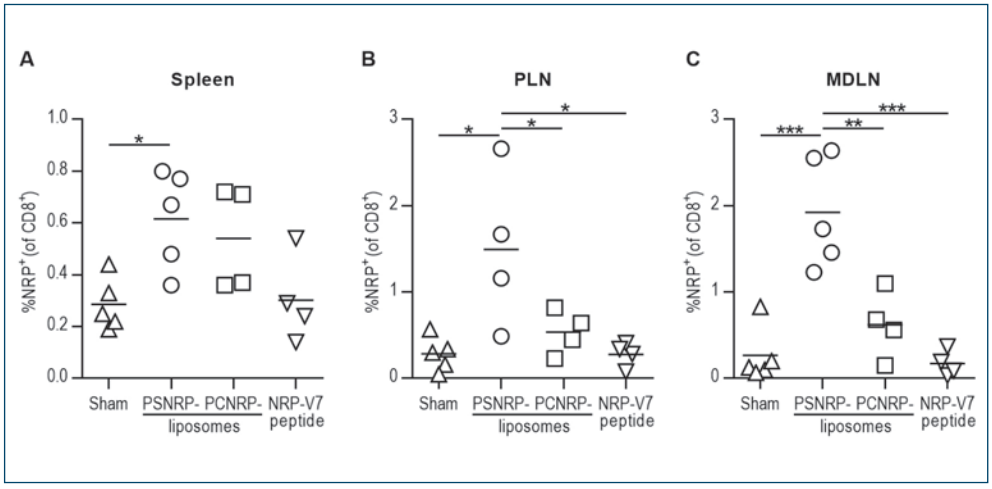


Figure 10. ***Antigen-specific CD8⁺ T cell expansion 4 days after i.p. administration of PS-liposomes to NOD mice.*** **(A)** *Detection of NRP⁺CD8⁺ T cells in the spleen,* **(B)** *PLN and* **(C)** *MDLN of NOD mice treated with PBS (n=5, sham, triangles), PSNRP-liposomes (n=4-5, circles), PCNRP-liposomes (n=4, squares) and NRP-V7 peptide diluted in PBS (n=4, upside-down triangles). Each symbol represents one animal and lines show the mean of all included mice, pooled from 4 independent experiments. Comparisons between groups showed significant differences (*p<0.05, **p<0.01, ***p<0.001, One-way ANOVA and Bonferroni multiple comparison test).*

4. PS-liposomes treatment displays optimal safety and tolerability in NOD mice

To study the safety of PS-liposomes treatment, 6 NOD mice received 14 daily i.p. injections of PS30-liposomes and were monitored for weight and several physical and behavioural traits. Additionally, 6 mice were administered with PBS as control. No animals were euthanised during the study due to severe complications of the treatment, and those mild complications exhibited by PS30-liposomes-treated animals were similar to those of the sham group (**Table 15**). Presence of glucosuria during and after the treatment was not detected in any of the animals either. As shown in **Figure 11**, the biochemical analysis of the sera showed similar values for all analytes in sham and PS30-liposomes group. With a particular interest for this project, total CH, HDL CH and triglycerides concentrations remained unchanged after 14 daily administrations of PS30-liposomes and within reference values for NOD mice (provided by the Mouse Phenome Database by Jackson Laboratories, phenome.jax.org). ALT remained in normal concentrations after PS30-liposomes treatment; however, urea concentration in both groups was found outside the reference values, meaning that this alteration could not be induced by PS30-liposomes treatment. Creatinine reference values were not reported, although both groups displayed similar concentrations.

Table 15. List of parameters found altered in NOD mice treated with PS30-liposomes

Parameter	Incidence	
	Sham	PS30-liposomes
Loss of 10% of body weight	1/6 (16%)	2/6 (33%)
Piloerection	0/6 (0%)	1/6 (16%)
Mild bruises on the abdomen	1/6 (16%)	0/6 (0%)
Mild bruises on the tail	1/6 (16%)	0/6 (0%)
Mild aggressive behaviour	0/6 (0%)	1/6 (16%)

Moreover, an assay of posology was carried out by administering one single i.p. dose of PS-liposomes with 3-fold-increased lipid concentration (90 mM, PS90-liposomes). A higher lipid concentration was not considered due to technical difficulties in producing and handling of the liposomes. None of the 3 prediabetic NOD mice treated with PS90-liposomes presented any mild nor severe complications derived from the PS-liposomes treatment administration, neither at 6 hours after the treatment nor after 7 days of follow-up. Moreover, the PS90-liposomes treated group did not show any differences in weight, physical or behavioural traits when compared to the control group.

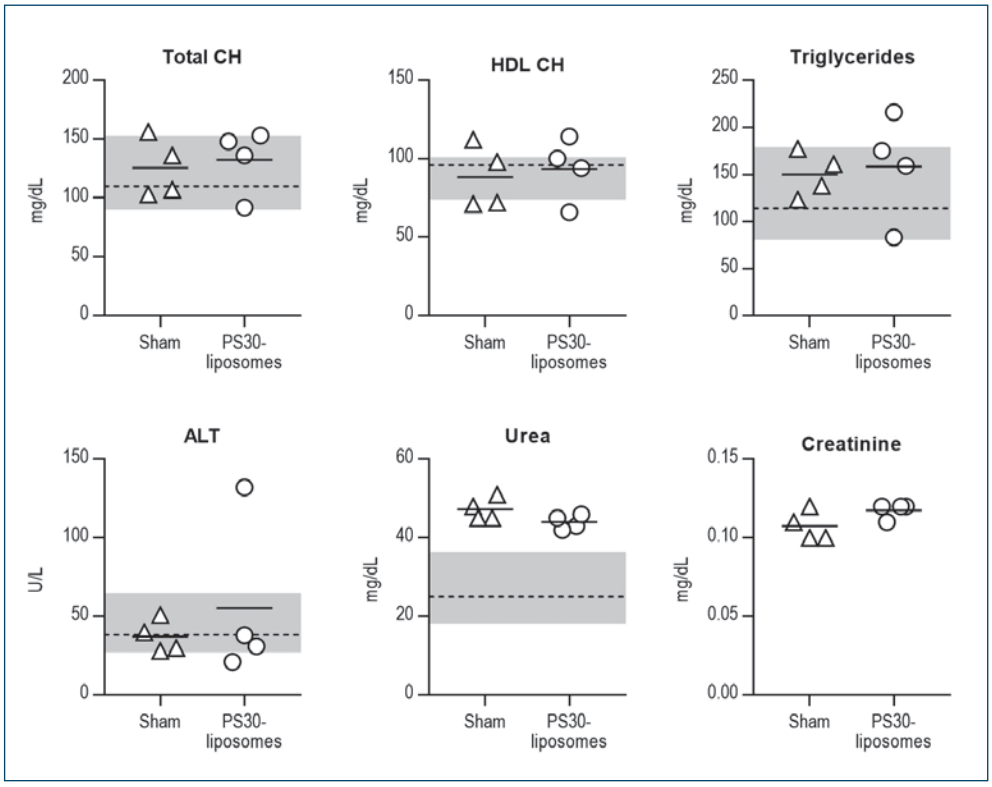


Figure 11. ***Serum biochemical parameters remain unchanged after 14 daily administrations of PS30-liposomes in NOD mice.*** *Concentrations of total CH, HDL CH, triglycerides, urea, creatinine (mg/dL) and ALT (U/L) in the sera of PBS-treated mice (n=4, triangles) and PS30-liposomes-treated mice (n=4, circles). Continuous lines represent the mean of all included mice. Grey bands are reference values of the different analytes for NOD mice, obtained from the Mouse Phenome Database by Jackson Laboratories (phenome.jax.org), and discontinuous lines represent the median values. Reference values for creatinine were not reported. Significant differences were not found between groups.*

SECTION II: PS-LIPOSOMES TREATMENT INDUCES TOLEROGENIC FEATURES IN DCs AND ARRESTS EAE PROGRESSION IN EAE-INDUCED C57BL/6 MICE

The second part of this work was focused on validating the immunomodulating and preventive potential of the PS-liposomes strategy in another autoimmune disease such as MS. To do so, we used the MOG_{40-55} peptide EAE-induced model in C57BL/6 mice. Therefore, DCs were derived from bone marrow precursors to study the phagocytosis kinetics of PS-liposomes and the tolerogenic effect that their engulfment prompted in DCs in terms of phenotype and PGE_2 secretion. Furthermore, the preventive effect of PSMOG-liposomes on the progression of EAE disease was determined, and $FoxP3^+$ and $FoxP3^-$ subsets and the intensity of the CD49b and LAG3 markers were assessed to discern the effect of PSMOG-liposomes administration in Treg cell subsets. The experiments performed for this section have been published in Pujol-Autonell *et al.*, 2017.

5. PS-liposomes show appropriate features, morphology and autoantigen encapsulation

PS-liposomes were produced with PS/PC/CH at 1:1:1.33 molar ratio and characterised. All types of PS-liposomes had a final lipid concentration of 30 mM, a diameter larger than 500 nm to guarantee efficient phagocytosis by DCs and a negative ζ-potential given by the presence of PS on their surface. A summary of their physicochemical features can be found in **Table 16**. Using cryo-TEM analysis, PSMOG-liposomes revealed a multivesicular vesicle morphology (**Figure 12**).

6. PS-liposomes are efficiently phagocyted by murine DCs

Murine DCs were cultured with 1 mM PSMOG-liposomes (PSMOG-DCs) to assess changes in their viability 24 hours after the addition of the stimuli. As controls, DCs were cultured in basal conditions (iDCs), with 1 mM empty PS-liposomes (PS-DCs), with 0.46 mg/mL MOG_{40-55} peptide (MOG-DCs), or with 100 ng/mL LPS to obtain mDCs. The viability of PSMOG-DCs was >80% (**Figure 13A**) in all experiments, and control DCs behaved similarly.

Next, a time course analysis was performed to assess capture kinetics of fluorescently-labelled PSOG488-liposomes by DCs differentiated from bone marrow precursor cells from EAE-induced C57BL/6 mice (**Figure 13B** and **Supplementary Figure 3**). At every checkpoint of the study, the amount of OG488 fluorescence was higher at 37 °C than at 4 °C (p<0.0001), thus demonstrating that DCs engulf PS-

Table 16. Physicochemical features of the liposomes used in SECTION II

Liposome nomenclature	Number of batches	Lipid concentration (mM)	Diameter size (nm)	PdI	ζ-potential (mV)	Encapsulation efficiency (%)
PS-liposomes	5	29.70 ± 0.46	985 ± 144	0.35 ± 0.05	–35.40 ± 8.44	—
PSOG488-liposomes	1	30.01	955	0.40	–41.10	—
PSMOG-liposomes	6	30.01 ± 0.06	861 ± 130	0.32 ± 0.19	–36.19 ± 5.32	91.53 ± 4.10

Data presented as mean ± SD. PdI: polydispersity index. From Pujol-Autonell *et al.*, 2017.

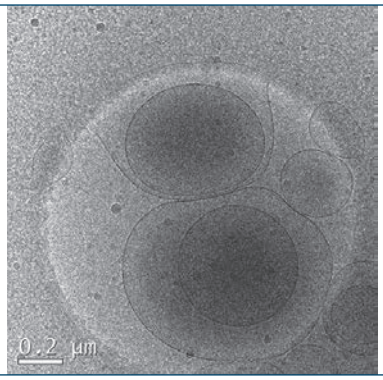


Figure 12. ***PSMOG-liposomes display multivesicular vesicle morphology.*** *Cryo-TEM image of PSMOG-liposomes. Bar = 0.2 µm. From Pujol-Autonell* et al.*, 2017.*

liposomes by an active mechanism of phagocytosis which cannot take place at a low temperature, in which DCs are metabolically inactive.

7. PS-liposomes induce a tolerogenic phenotype and functionality in murine DCs

To assess the tolerogenic potential of PSMOG-liposomes in cultured DCs, the expression of antigen-presenting (MHC class II) and costimulatory molecules (CD86 and CD40) was analysed 24 hours after the addition of the stimuli. First, iDCs always displayed lower expression of the three markers than mDCs, as expected (**Figure 14A-C**). PSMOG-liposomes capture did not alter the expression of MHC class II in comparison to iDCs, PS-DCs and MOG-DCs, but its expression was decreased when compared to mDCs ($p<0.05$). Regarding costimulatory molecules, PSMOG-DCs showed lower CD86 levels and higher CD40 levels than iDCs, similar to those of PS-DCs and MOG-DCs, and always lower than those of mDCs ($p<0.05$).

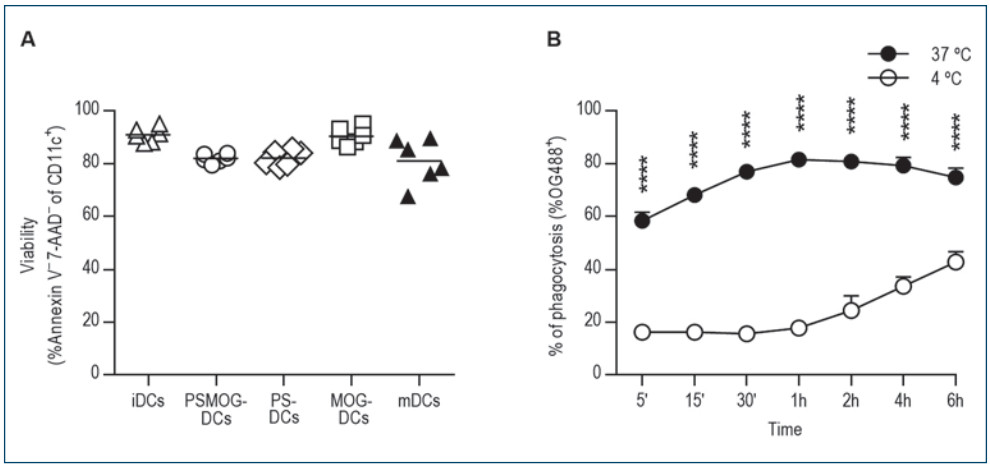


Figure 13. ***PS-liposomes do not affect the viability of murine DCs and are rapidly captured.*** **(A)** *DCs viability assessed by annexin V and 7-AAD staining. White symbols represent iDCs (basal conditions, triangles), DCs after the engulfment of PSMOG-liposomes (PSMOG-DCs, circles), after the uptake of empty PS-liposomes (PS-DCs, rhombus) or after the capture of* MOG_{40-55} *peptide (MOG-DCs, squares), 24 hours after culture; black triangles represent mDCs, 24 hours after culture with LPS. Data obtained from 6 independent experiments.* **(B)** *Time course of the capture of PSOG488-liposomes by DCs from EAE-induced C57BL/6 mice at 37 °C (black circles) and at 4 °C (white circles). Results are mean ± SD of three independent experiments (*****p<*0.0001, Two-way ANOVA). From Pujol-Autonell* et al.*, 2017.*

These changes point to the acquisition of a semimature phenotype by PSMOG-DCs after the capture of PSMOG-liposomes.

Additionally, PGE_2 secretion by DCs was determined in culture supernatants. Based on the literature (Fadok *et al.*, 1998) and previous findings by the group (Pujol-Autonell *et al.*, 2013, 2015), PGE_2 is known to have a relevant role in the induction of tolerance by APCs after efferocytosis of apoptotic cells and, also, after engulfment of PS-liposomes. Thus, DCs were cultured with the different stimuli for 24 hours to collect culture supernatants. As shown in **Figure 14D**, the concentration of PGE_2 in PSMOG-DCs culture was significantly higher than the found in iDCs or MOG-DCs supernatant (p<0.05), and showed a trend to be also increased when compared to PS-DCs.

8. PS-liposomes treatment reduces the incidence and the severity of EAE disease

C57BL/6 mice induced with EAE disease were treated with two doses of PSMOG-liposomes at days 5 and 9 after induction to test the preventive effect of the therapy. PBS, empty PS-liposomes and MOG_{40-55} diluted in PBS were administered on

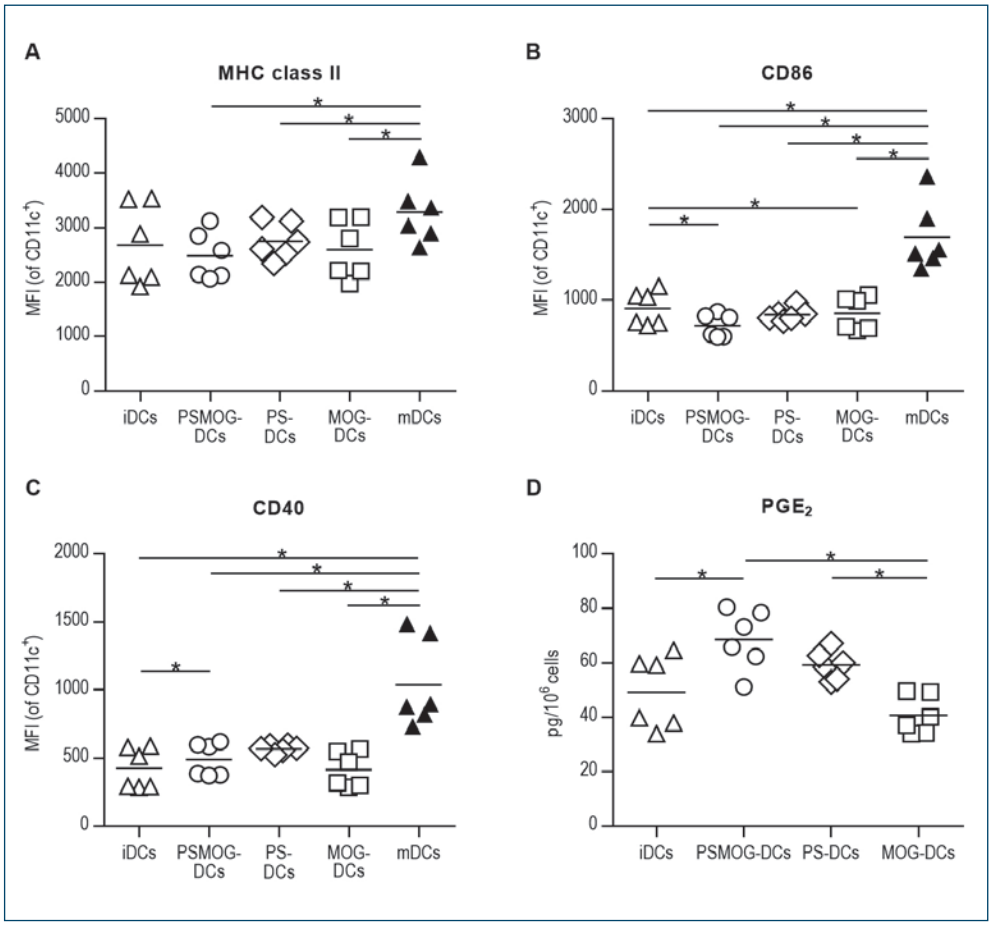


Figure 14. ***Capture of PS-liposomes induces a tolerogenic phenotype and increased PGE_2 secretion in murine DCs.*** **(A-C)** *MFI for MHC class II, CD86 and CD40 molecules in DCs from EAE-induced C57BL/6 mice, and* **(D)** *quantification of PGE_2 (pg/10^6 cell) released by DCs into the cell culture supernatant. White symbols represent iDCs (basal conditions, triangles), DCs after the engulfment of PSMOG-liposomes (PSMOG-DCs, circles), after the uptake of empty PS-liposomes (PS-DCs, rhombus) or after the capture of MOG_{40-55} peptide (MOG-DCs, squares), 24 hours after culture; black triangles represent mDCs, 24 hours after culture with LPS. Lines show the mean of six independent experiments, and comparisons between groups showed significant differences (*p<0.05, Wilcoxon test). From Pujol-Autonell I* et al.*, 2017.*

the same days as controls. The effect of each treatment was measured in terms of incidence, cumulative score (sum of the daily EAE score for each mouse), maximum clinical score (the most severe score presented by each mouse), disease onset day and overall survival until the end of the study (**Table 17**). Thus, mice treated with PSMOG-liposomes displayed a significantly lower incidence of EAE disease (p<0.05) and lower cumulative score (p<0.01) than the sham and PS-liposomes

Table 17. Clinical features of the EAE disease in treated and control mice

Treatment	Incidence	Cumulative score	Maximum score	Onset day	Survival
Sham	12/13 (92.31%)*	42.19 ± 24.99**	2.83 ± 1.94	13.17 ± 4.73	10/13 (76.92%)
PSMOG-liposomes	5/11 (45.45%)	14.36 ± 17.89	1.41 ± 1.59	16.00 ± 4.56	11/11 (100%)
PS-liposomes	10/10 (100%)*	45.60 ± 23.61**	3.90 ± 1.70**	14.80 ± 2.70	8/10 (80%)
MOG_{40-55} peptide	6/8 (75%)	25.88 ± 21.48	2.38 ± 1.77	15.50 ± 2.35	8/8 (100%)

Data expressed as mean ± SD. Clinical score of EAE disease was followed-up daily and measured according to the criteria: 0, asymptomatic; 0.5, loss of distal half of tail tone; 1, loss of entire tail tone; 1.5, hind limb weakness; 2, hind limb paralysis; 2.5, hind limb paraplegia; 3, forelimb weakness; 4, quadriparesis; 4.5, severe quadriparesis; 5, quadriplegia and 6, death. Comparisons between PSMOG-liposomes-treated group vs sham-treated and PS-liposomes-treated groups showed significant differences (*p<0.05, Fisher's Exact test; **p<0.01, Mann-Whitney test). From Pujol-Autonell *et al*., 2017.

group. As for the maximum score, that of PSMOG-liposomes group was significantly decreased in comparison to that of PS-liposomes group (p<0.01), and the same trend was observed when comparing the maximum score of sham and MOG_{40-55} peptide groups with that of PSMOG-liposomes group. The onset day of EAE disease in PSMOG-liposomes group appeared to be slightly delayed when compared to that of the other groups, although significant differences were not found. Regarding mice survival until the end of the study, no differences were found in survival percentages between the groups, but only mice belonging to the sham and PS-liposomes groups had to be euthanised upon reaching a clinical score of 6 before the end of the follow-up.

Finally, mice treated with PSMOG-liposomes showed a daily EAE score significantly lower than sham and PS-liposomes treated groups (p≤0.05, **Figure 15A**). This is also reflected when comparing the area under the curve (AUC) of the clinical score of each treatment (**Figure 15B**), where PSMOG-liposomes group displayed a lower AUC than sham and PS-liposomes groups (p<0.01), and a tendency to be lower than that of MOG_{40-55} peptide group. Also, the group treated with MOG_{40-55} peptide exhibited a lower clinical score AUC than the PS-liposomes group's (p<0.05) and a tendency to be lower than the sham group's, but not as reduced as the PSMOG-liposomes group's. In addition, EAE-induced mice treated with PSMOG-liposomes did not suffer a decrease in their body weight as mice treated with PS-liposomes, MOG_{40-55} peptide or PBS underwent (p<0.001, **Figure 15C**). Supporting this, PSMOG-liposomes had a greater weight AUC than PS-liposomes group (p<0.05, **Figure 15D**).

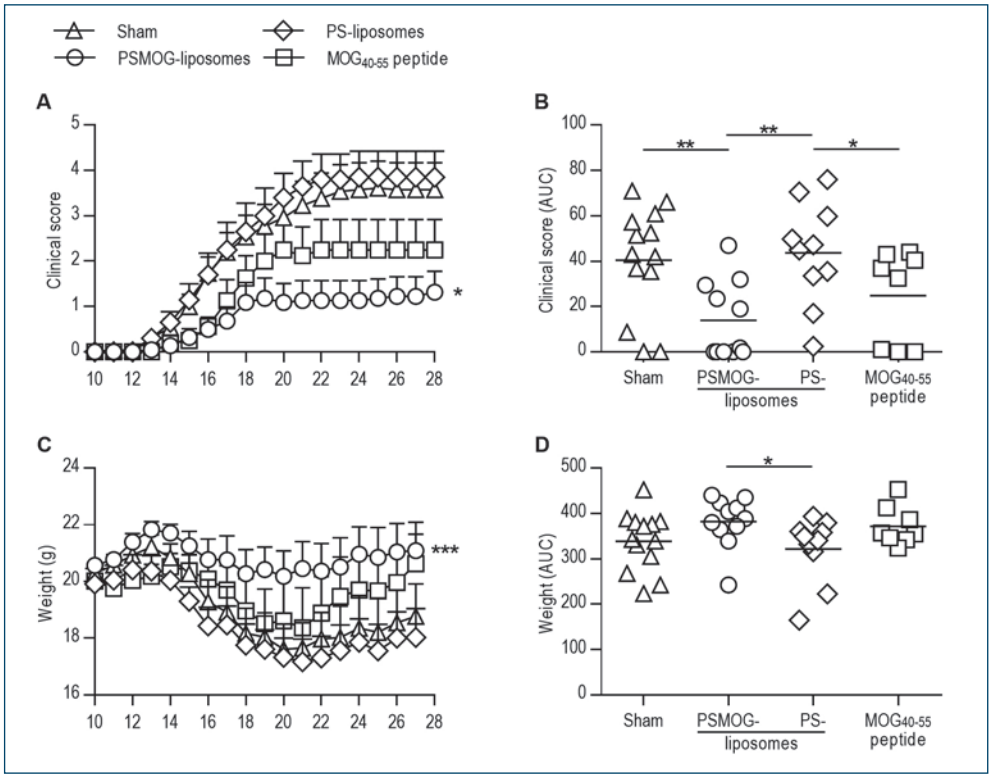


Figure 15. ***PS-liposomes treatment arrests the autoimmune reaction induced in the EAE model.*** **(A)** *Clinical score of EAE disease according to the criteria: 0, asymptomatic; 0.5, loss of distal half of tail tone; 1, loss of entire tail tone; 1.5, hind limb weakness; 2, hind limb paralysis; 2.5, hind limb paraplegia; 3, forelimb weakness; 4, quadriparesis; 4.5, severe quadriparesis; 5, quadriplegia; and 6, death. Follow-up was performed blindly in mice treated with PBS (sham, triangles, n=13), PSMOG-liposomes (circles, n=11), PS-liposomes (rhombus, n=10) or MOG$_{40-55}$ peptide (squares, n=8). Results are mean ± SEM from two independent experiments. Significant differences were found when comparing PSMOG-liposomes group with PS-liposomes and sham groups (*p<0.05, Mann-Whitney test).* **(B)** *AUC of clinical score curves; lines show the mean of all included mice. Comparison between groups revealed differences (*p<0.05, **p<0.01, Mann-Whitney test).* **(C)** *Mean weight in EAE-induced mice after treatment with PBS (sham, triangles, n=13), PSMOG-liposomes (circles, n=11), PS-liposomes (rhombus, n=10) or MOG$_{40-55}$ peptide (squares, n=8). Results are mean ± SEM from two independent experiments. Significant differences were found when comparing PSMOG-liposomes group with all control groups (***p<0.001, Mann-Whitney test).* **(D)** *AUC of the weight of the animals; lines show the mean of all included mice. Comparison between groups revealed differences (*p<0.05, Mann-Whitney test). From Pujol-Autonell* et al.*, 2017.*

9. Treatment with PS-liposomes induces an increase of putative TR1 cells

In order to find the T cell subsets responsible for the beneficial effect of PSMOG-liposomes therapy, classical $CD3^+CD4^+CD25^+FoxP3^+$ Treg cells and non-classical TR1 cells were analysed in the spleen at day 6 after PS-liposomes treatment, mean-

ing day 15 after EAE-induction (**Figure 16** and **Figure 17**, respectively). FoxP3 intracellular staining (**Supplementary Figure 4**) revealed that PSMOG-liposomes treatment significantly decreased the percentage of classical $CD25^+FoxP3^+$ (gated in $CD3^+CD4^+$) Treg cells when compared to sham group ($p<0.05$, **Figure 16A**). Nonetheless, it is worth noting that the frequency of $CD25^+FoxP3^-$ T cells increased when compared to their sham counterparts ($p<0.05$, **Figure 16B**). In fact, the percentage of $CD25^+FoxP3^-$ T cells in PSMOG-liposomes-treated mice correlated inversely with the clinical score at day 15 after EAE-induction (Spearman's r = –0.9258, $p<0.0001$), as shown in **Figure 16C**, but no correlation was observed between these

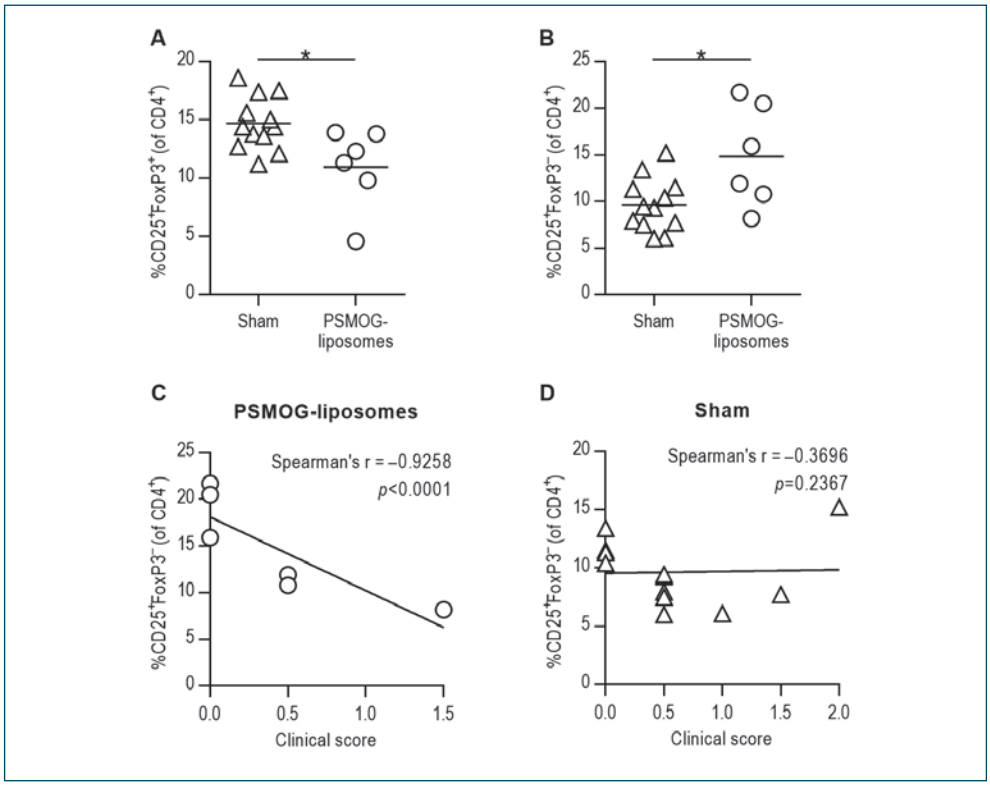


Figure 16. ***Treatment with PS-liposomes causes an increase in the percentage of $CD25^+FoxP3^-CD4^+$ T cells that correlates with milder EAE disease form.*** **(A)** *Percentage of classical Treg cells ($CD25^+FoxP3^+$, gated in $CD3^+CD4^+$) and* **(B)** *$CD25^+FoxP3^-$ (gated in $CD3^+CD4^+$) cells in the spleen of sham control group (triangles, n=12) and PSMOG-liposomes treated mice (circles, n=6) at day 15 after induction of EAE. Lines show the mean of 6-12 mice. Comparisons between groups showed significant differences (*p<0.05, Mann-Whitney test).* **(C)** *Correlation between the clinical score at day 15 after induction of EAE and percentage of $CD25^+FoxP3^-$ cells in PSMOG-liposomes treated mice (n=6, Spearman's r = –0.9258,* p<*0.0001, Spearman's correlation analysis).* **(D)** *Correlation between the clinical score at day 15 after induction of EAE and percentage of $CD25^+FoxP3^-$ cells in sham-treated mice (n=12, Spearman's r = –0.3696,* p=*0.2367, Spearman's correlation analysis). From Pujol-Autonell* et al.*, 2017.*

parameters in the sham group (**Figure 16D**). In accordance with the percentages shown, the absolute count of $CD4^+CD25^+FoxP3^+$ T cells tended to decrease in PSMOG-liposomes group ($6.31 \times 10^5 \pm 2.03 \times 10^5$, mean ± SD) when compared to sham group ($11.05 \times 10^5 \pm 0.76 \times 10^5$), but no tendency was observed when comparing the absolute numbers of $CD4^+CD25^+FoxP3^-$ T cells in PSMOG-liposomes group ($9.13 \times 10^5 \pm 4.58 \times 10^5$) and sham group ($8.95 \times 10^5 \pm 1.71 \times 10^5$).

Since $CD4^+CD25^+FoxP3^-$ T cells could potentially belong to the TR1 subset, the expression of TR1 cell markers CD49b and LAG3 was next examined on splenocytes of the sham and PSMOG-liposomes groups (**Figure 17** and **Supplementary Figure 5**). Even though no significant differences were found in CD49b expression when comparing both groups, a slight tendency for higher CD49b expression was observed in the PSMOG-liposomes group. Also supporting this trend, LAG3 expression was significantly higher in PSMOG-liposomes group when compared to sham group ($p<0.05$).

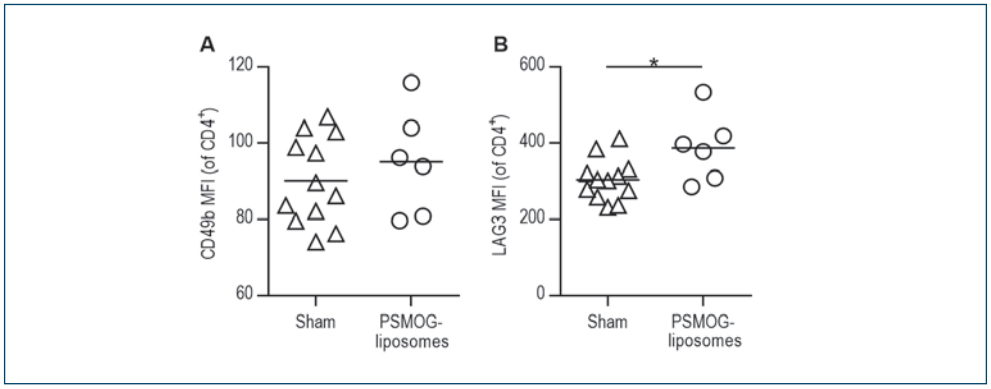


Figure 17. ***$CD4^+$ T cells in PS-liposomes-treated mice show higher LAG3 expression than their sham counterparts.*** **(A)** *MFI of CD49b and* **(B)** *LAG3 in $CD3^+CD4^+$ T cells in the spleen of sham control group (triangles, n=12) and PSMOG-liposomes-treated mice (circles, n=6) at day 15 after induction of EAE. Lines show the mean of 6-12 mice. Comparisons between groups showed significant differences (*p<0.05, Mann-Whitney test). From Pujol-Autonell* et al.*, 2017.*

SECTION III: PHAGOCYTOSIS OF PS-LIPOSOMES DRIVES IMMUNOREGULATION IN DCs FROM ADULT AND PAEDIATRIC SUBJECTS WITH T1D

Finally, the third part of this work aimed at determining the effects of the PS-liposomes therapy on human DCs obtained from patients with T1D. To that end, DCs from adult subjects were cocultured with PS-liposomes encapsulating the A and B chains of the human insulin molecule, which contain well-known β-cell-specific target epitopes in human T1D. We analysed the changes in viability, liposome capture kinetics, phenotype, soluble molecule secretion, ability to induce autologous T cell proliferation and transcriptome, discovering that phagocytosis of PS-liposomes is also able to prompt tolerogenic features in these DCs. These data have been published in Rodriguez-Fernandez *et al.*, 2018. Furthermore, since T1D is mainly diagnosed in children and adolescents, the induction of tolerance caused by PS-liposome capture was tested in DCs obtained from paediatric patients with recently-diagnosed T1D and with established disease. In assessing the capture kinetics of PS-liposomes, we observed that DCs from paediatric patients with established T1D phagocyted PS-liposomes in a slower and less efficient manner than their counterparts correlating with the time of disease progression. Nevertheless, DCs from both subsets of patients displayed a tolerogenic phenotype and gene expression profile after the uptake of PS-liposomes. Therefore, these results reinforce the potential of this immunotherapy as a promising strategy to arrest autoimmune aggression in human T1D and open the door to find ways in which to optimise its therapeutic effect and clinical benefit.

10. PS-liposomes efficiently encapsulate human insulin peptides and display multivesicular vesicle morphology

For this set of experiments, both PS-liposomes and PC-liposomes were generated and characterised. We intended for all liposomes batches to have a final lipid concentration of 30 mM and a minimum diameter of 500 nm to ensure their phagocytosis by DCs. The liposomes presented a negative ζ-potential when having PS in their composition whereas, in its absence, the liposomes exhibited a more neutral surface charge. **Table 18** shows the characteristics of the liposomes used in SECTION III. Of note, differences between encapsulation efficiencies of human A and B insulin peptides within PS-liposomes are due to amino acid composition and different solubility of the chains in PBS media. Additionally, B chain is more positively charged (0.1 mV, **Table 4**) than A chain (–2.3 mV) at neutral pH, contributing to a higher encapsulation efficiency in negatively-charged PS-liposomes. Also, cryo-TEM analysis revealed the multilamellar and multivesicular vesicle morphology of PS-liposomes (**Figure 18**).

Table 18. Physicochemical features of the liposomes used in SECTION III

Liposome nomenclature	Number of batches	Lipid concentration (mM)	Diameter size (nm)	PdI	ζ-potential (mV)	Encapsulation efficiency (%)
PSOG488-liposomes	4	30.00 ± 0.01	836 ± 217	0.32 ± 0.06	–38.90 ± 2.52	—
PCOG488-liposomes	4	30.01 ± 0.01	1,665 ± 488	0.32 ± 0.09	–7.60 ± 2.68	—
PSA-liposomes	4	30.11 ± 0.19	732 ± 86	0.39 ± 0.23	–38.25 ± 5.56	53.04 ± 32.13
PSB-liposomes	4	30.11 ± 0.19	838 ± 238	0.52 ± 0.35	–37.65 ± 5.86	94.90 ± 3.48

Data presented as mean ± SD. PdI: polydispersity index.

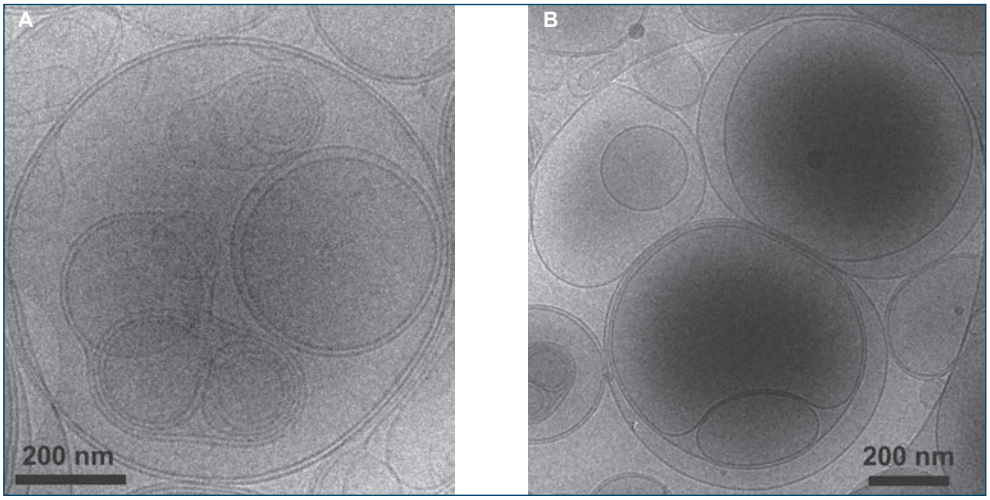


Figure 18. ***PSA-liposomes and PSB-liposomes display multilamellar and multivesicular vesicle morphology.*** *Cryo-TEM image of* **(A)** *PSA-liposomes and* **(B)** *PSB-liposomes. Bar = 0.2 μm. From Rodriguez-Fernandez* et al.*, 2018.*

11. Adult patients with T1D and control subjects display similar features in terms of gender, age and BMI

In order to study the changes in DCs caused by phagocytosis of PS-liposomes, 34 patients with T1D (50% female) from the Germans Trias i Pujol Hospital and 24 control subjects (46% female) were recruited to donate a 50 mL blood sample. All subjects met the inclusion and exclusion criteria and gave informed consent to participate in the study. Their clinical data is summarised in **Table 19**, and no differences were found in the parameters analysed.

Table 19. Data from adult patients with T1D and control subjects recruited for the study

Parameter	Control subjects	Patients with T1D	*p*-value
N	24	34	—
Gender	11/24 (46%) Female 13/24 (54%) Male	17/34 (50%) Female 17/34 (50%) Male	—
Age (years)	30.46 ± 8.18	32.54 ± 8.96	0.4301
BMI (kg/m^2)	23.90 ± 2.87	23.80 ± 3.11	0.9075
Age at T1D diagnosis (years)	NA	20.79 ± 9.90	—
Duration of T1D (years)	NA	11.75 ± 9.70	—
HbA1c (%)	NP	7.66 ± 1.26	—
HbA1c (mmol/mol)	NP	60.22 ± 13.73	—

Data presented as mean ± SD; *p*-value calculated from Mann-Whitney test. BMI: body mass index; HbA1c: glycated haemoglobin; NA: not applicable; NP: not performed. From Rodriguez-Fernandez *et al.*, 2018.

12. The efficiency of monocyte to DC differentiation is similar in adult patients with T1D and control subjects

Monocytes were isolated from PBMCs to differentiate DCs. **Table 20** shows the yield of monocyte isolation —percentage 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 and viability of the isolated cells, and their differentiation efficiency into DCs —expressed as the purity of CD11c marker— at day 6 of cell culture. No statistically significant differences were found when comparing these parameters between both groups.

13. PS-liposomes phagocytosis by adult DCs is performed with optimal kinetics and without affecting the viability

The viability of the different conditions of DCs (iDCs, PSAB-DCS, mPSAB-DCs and mDCs) was analysed to determine PSAB-liposomes toxicity. DCs from both control subjects (**Figure 19A**) and patients with T1D (**Figure 19B**) showed an average viability higher than 90% in each condition, thus meaning that PS-liposomes at 1 mM are not toxic to DCs.

Moreover, a time course experiment was performed to determine the PS-liposomes uptake kinetics by DCs from both adult control subjects and patients with T1D (**Figure 19C-D**). To do so, DCs were cocultured with PSOG488-liposomes at 37 °C and OG488 fluorescence was assessed at different time points. A control phagocytosis assay was performed at 4 °C, temperature at which DCs are metabolically inactive and unable to phagocyte. As shown in **Figure 19C**, the capture of PSOG488-

Table 20. Data from monocyte's isolation and differentiation into DCs of the adult subjects

Parameter	Control subjects	Patients with T1D	*p*-value
Yield (day 1, %)	54.95 ± 24.97	56.62 ± 18.12	0.3789
CD14 purity (day 1, %)	80.59 ± 10.18	79.33 ± 7.56	0.2286
Viability (day 1, %)	95.06 ± 4.14	94.92 ± 3.60	0.3664
Differentiation efficiency (CD11c purity, day 6, %)	87.96 ± 6.61	86.92 ± 6.86	0.5766

Data presented as mean ± SD; *p*-value calculated from Mann-Whitney test. Yield: percentage of the absolute number of $CD14^+$ cells in the positively isolated fraction related to the absolute number of $CD14^+$ cells in total PBMCs. CD14 purity: percentage of $CD14^+$ cells in the isolated fraction. Viability: percentage of Annexin V^-7-AAD^- cells of isolated $CD14^+$ cells. Differentiation efficiency: percentage of $CD11c^+$ cells. From Rodriguez-Fernandez *et al.*, 2018.

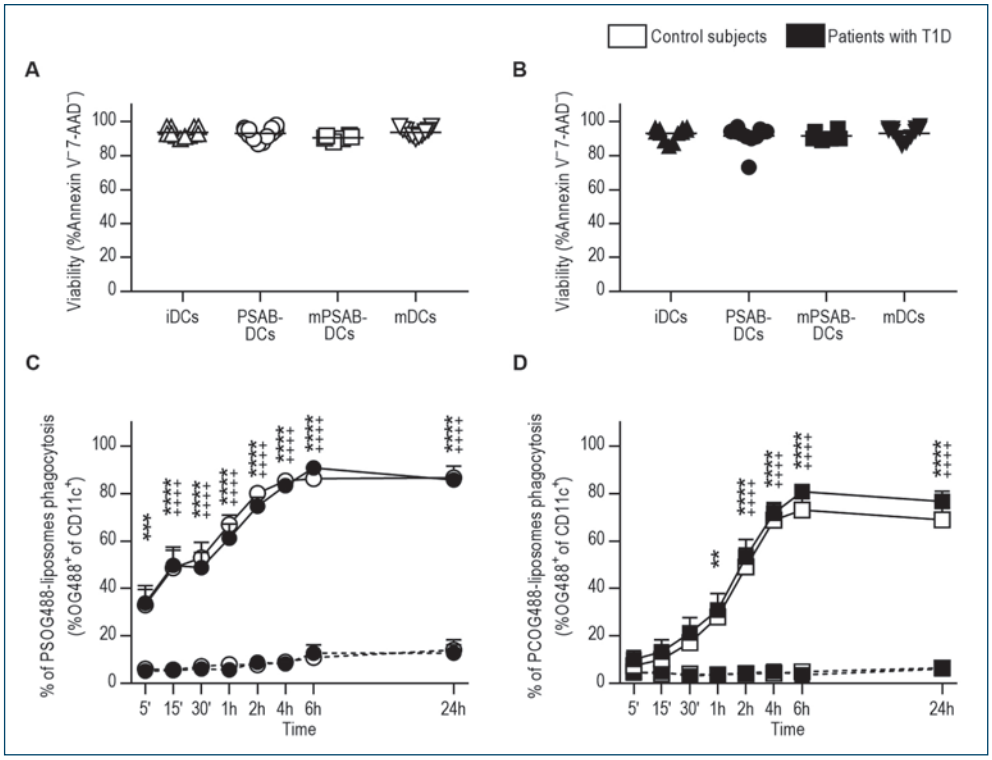


Figure 19. ***PS-liposomes preserve high viability in DCs and are efficiently phagocyted.*** **(A)** *Viability of DCs from control subjects (white symbols, n≥6) and* **(B)** *patients with T1D (black symbols, n≥6) assessed by annexin V and 7-AAD staining. Triangles, iDCs; circles, iDCs cultured with PSAB-liposomes (PSAB-DCs); squares, PSAB-DCs matured with CC (mPSAB-DCs); upside-down triangles, mDCs induced by CC, 24 hours after culture. Lines show the mean of all samples.* **(C)** *Time course of the uptake of PSOG488-liposomes (circles) and* **(D)** *PCOG488-liposomes (squares) by DCs obtained from control subjects (white symbols, n=5-6) and patients with T1D (black symbols, n=9-10) at 37 °C (continuous line) and at 4 °C (discontinuous line). Results are mean ± SEM. Comparisons between phagocytosis by control subjects' DCs at 37 °C and 4 °C showed significant differences (++++p<0.0001, Two-way ANOVA); also, significant differences were found when comparing phagocytosis in patients with T1D at 37 °C and 4 °C (**p<0.01, ***p<0.001, ****p<0.0001, Two-way ANOVA). No differences were found when comparing the results of control subjects and patients with T1D (Two-way ANOVA). From Rodriguez-Fernandez* et al.*, 2018.*

liposomes by DCs from patients with T1D was significantly higher at 37 °C than at 4 °C at each checkpoint (p<0.001), and control subjects DCs' showed the same differences starting at 15 minutes of coculture (p<0.0001). These results prove that human DCs engulf PS-liposomes by an active mechanism of phagocytosis. Also, PS-liposomes uptake kinetics was identical between control subjects and patients.

To indirectly assess the role of PS in the process of phagocytosis of PS-liposomes, the same experiment was performed replacing PSOG488-liposomes with PCOG488-liposomes (**Figure 19D**). The percentages of PC-liposomes phagocytosis by DCs from control subjects and patients with T1D were significantly higher at 37 °C than at 4 °C starting at 2 hours of coculture (p<0.001). Moreover, the kinetics of PC-liposomes capture did not differ between control subjects and patients.

When comparing the uptake kinetics of PS-liposomes with that of PC-liposomes in both subsets of subjects, statistically significant differences were found, as expected (**Supplementary Figure 6**). The presence of PS significantly accelerated the phagocytosis of PS-liposomes in the first 2 hours of coculture (p<0.05). Moreover, in preliminary experiments, both PS-liposomes and PC-liposomes phagocytosis were tested with differently-sized liposomes (diameter range 505-2,138 nm). As shown in **Supplementary Figure 7**, all PS-liposomes were phagocyted with similar kinetics in the 2 hours of coculture, independently of liposome size. All PC-liposomes were also phagocyted with comparable kinetics regardless of their size, thereby confirming that PS is the key factor in accelerating liposome phagocytosis.

14. PS-liposomes phagocytosis regulates the phenotypic maturation and functionality of human adult DCs

DCs from control subjects and patients with T1D were cocultured for 24 hours with PSAB-liposomes to assess changes in their phenotype. First, the expression of PS receptors, such as CD36, TIM-4 and integrin αvβ5, was analysed to confirm that their expression was modulable depending on the stimuli added to the culture. As shown in **Figure 20**, iDCs from both control subjects and patients expressed the three studied PS receptors, and the expression of CD36 and TIM-4 was downmodulated in mDCs in comparison to iDCs (p<0.05), whereas integrin αvβ5 only showed a tendency. Upon PSAB-liposomes uptake, PSAB-DCs from patients with T1D decreased CD36 expression (p<0.05) and upregulated TIM-4 expression (p<0.05) in comparison to iDCs. Furthermore, the expression of both CD36 and TIM-4 in PSAB-DCs was higher than in mDCs in both control subjects and patients with T1D (p<0.05), although the expression of integrin αvβ5 was higher in PSAB-DCs than in mDCs only in patients (p<0.05). Finally, PSAB-DCs from patients had increased levels of TIM-4 expression in comparison to PSAB-DCs from control subjects (p<0.05).

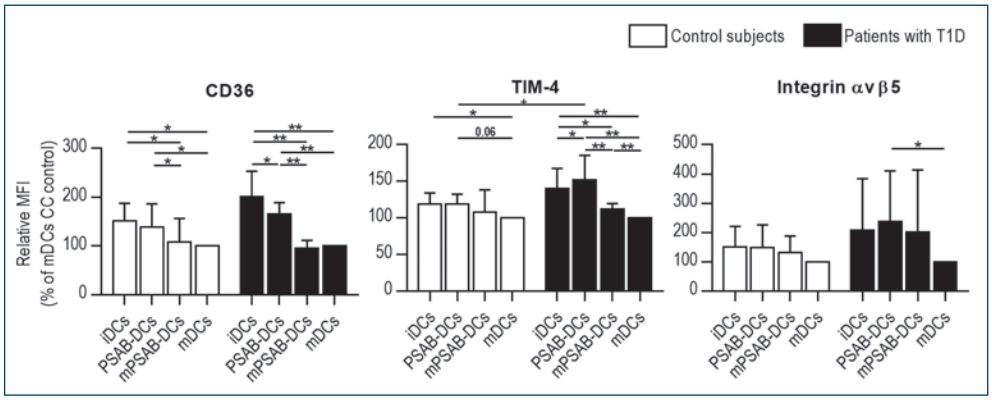


Figure 20. ***PS' receptors expression can be modulated in DCs by different stimuli.*** *Membrane expression of CD36, TIM-4 and integrin αvβ5 in DCs obtained from control subjects (white bars, n≥5) and patients with T1D (black bars, n≥8). Bars represent iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), PSAB-DCs matured with CC (mPSAB-DCs), or mDCs (induced by CC), 24 hours after culture. Data presented as mean ± SD of relative MFI, this being MFI of each culture condition referred to their respective mDCs control. Significant differences were found when comparing culture conditions in the same group of subjects (*p≤0.05, **p<0.01, Wilcoxon test), and when comparing the same culture condition between control subjects and patients with T1D (+p<0.05, Mann-Whitney test). From Rodriguez-Fernandez* et al.*, 2018.*

Afterwards, the expression of several molecules involved in the immunological functions of DCs was analysed (**Figure 21**). The membrane molecules assessed were: antigen-presenting molecules (HLA-ABC and HLA-DR), adhesion molecules (CD54), costimulatory molecules (CD40 and CD86), activation molecules (CD25), chemokine receptors (CCR7, CCR2 and CXCR4), and pattern recognition receptors (TLR2, CD14 and DC-SIGN). Regarding HLA molecules, HLA-ABC was expressed similarly in iDCs and mDCs from both groups, and decreased in PSAB-DCs from patients ($p<0.05$). Although significance was not reached, PSAB-DCs from control subjects displayed the same tendency. As for HLA-DR, iDCs showed a lower expression of this marker when compared to mDCs ($p<0.001$), and these levels were preserved in PSAB-DCs and mPSAB-DCs in patients. Also, they were lower when compared to those of mDCs ($p<0.01$). However, PSAB-DCs from control subjects showed higher expression of HLA-DR than iDCs, but lower than mPSAB-DCs and mDCs ($p<0.01$). Regarding the expression of the adhesion molecule CD54, this was lower in iDCs from patients in comparison to mDCs ($p<0.05$), and control subjects displayed the same tendency. In PSAB-DCs, no changes in CD54 expression were observed when compared to iDCs, but mDCs displayed increased levels of this molecule in comparison to PSAB-DCs ($p<0.05$). The expression of CD54 was higher in mPSAB-DCs than in PSAB-DCs ($p<0.05$), and similar to that of mDCs. Expression of costimulatory molecules CD86 and CD40 was lower in iDCs than in mDCs in both

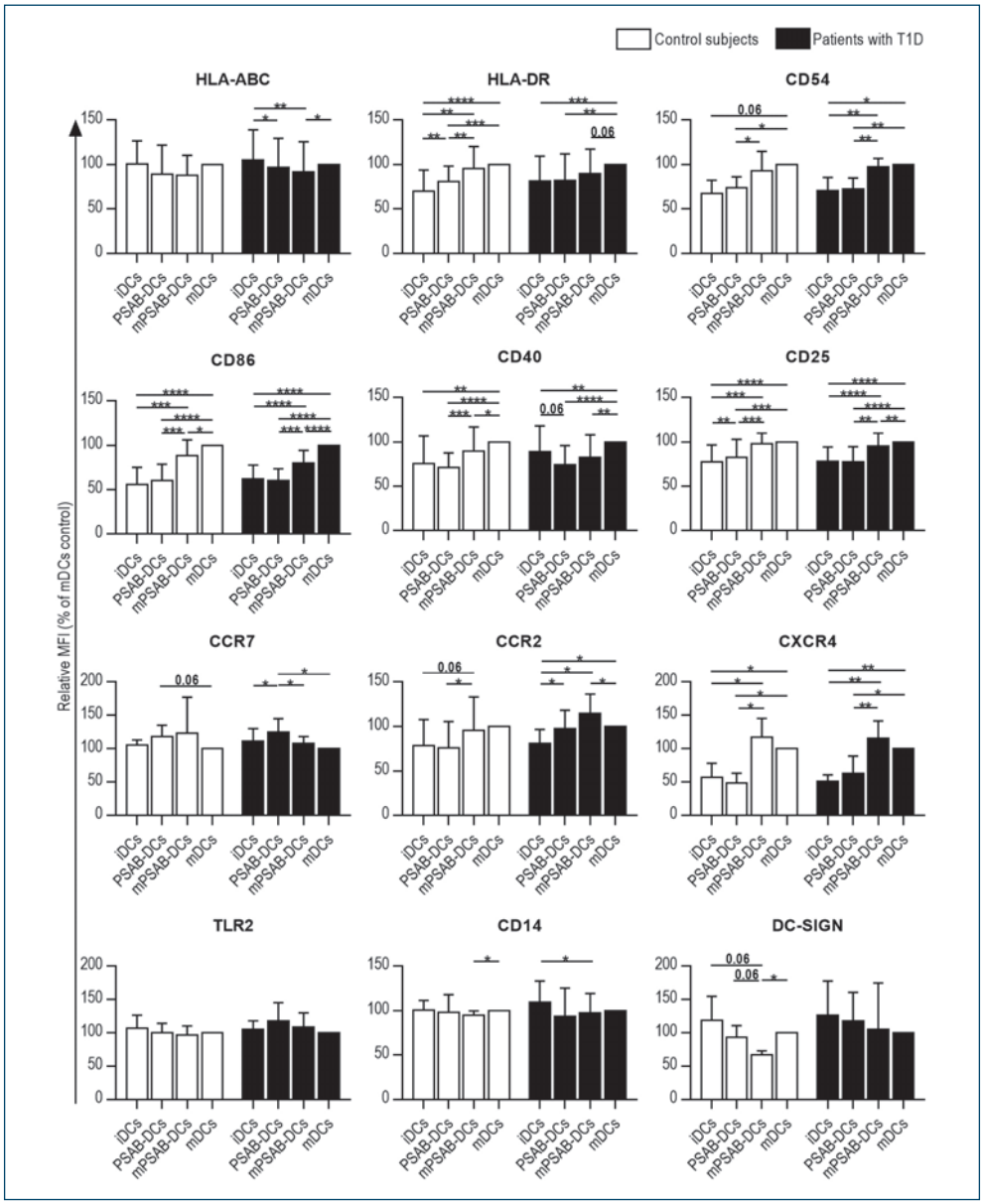


Figure 21. ***Capture of PS-liposomes regulates the immune-related molecules' phenotype in DCs.*** *Membrane expression of HLA-ABC, HLA-DR, CD54, CD86, CD40, CD25, CCR7, CCR2, CXCR4, TLR2, CD14 and DC-SIGN in DCs obtained from control subjects (white bars, n≥5) and patients with T1D (black bars, n≥8). Bars represent iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), PSAB-DCs matured with CC (mPSAB-DCs), or mDCs (induced by CC), 24 hours after culture. Data presented as mean ± SD of relative MFI, this being MFI of each culture condition referred to their respective mDCs control. Significant differences were found when comparing culture conditions in the same group of subjects (*p≤0.05, **p<0.01, ***p<0.001, ****p<0.0001, Wilcoxon test), and differences were not found when comparing the same culture condition between control subjects and patients with T1D. From Rodriguez-Fernandez* et al.*, 2018.*

groups of subjects (p<0.01). PSAB-liposomes uptake did not increase the expression of these molecules in PSAB-DCs in comparison to iDCs. Moreover, PSAB-DCs presented lower levels of these markers when compared to mDCs (p<0.0001), and even mPSAB-DCs displayed reduced levels of CD86 and CD40 in comparison to mDCs (p<0.05). Regarding the expression of CD25, it was lower in iDCs when compared to mDCs (p<0.0001). PSAB-DCs from control subjects exhibited an upregulation of CD25 in comparison to iDCs (p<0.01), whereas expression of CD25 in PSAB-DCs from patients with T1D remained unaltered. PSAB-DCs from both groups presented a downmodulation of CD25 when compared to mDCs (p<0.001). Furthermore, in patients with T1D, mPSAB-DCs displayed lower levels of CD25 than mDCs (p<0.01).

Chemokine receptors CCR7 and CCR2 were expressed in iDCs, and their expression increased in PSAB-DCs from patients (p<0.05). CCR7 was upregulated in PSAB-DCs when compared to mDCs in patients with T1D (p<0.05), and the same tendency was observed in control subjects. As for CCR2, mPSAB-DCs showed the highest expression of this molecule in patients (p<0.05), and the same pattern was determined in control subjects. CXCR4 was overexpressed in mDCs in comparison to iDCs in both groups (p<0.05) and was maintained low after PSAB-liposomes engulfment in PSAB-DCs. The expression of CXCR4 was higher in mPSAB-DCs in comparison to iDCs and PSAB-DCs (p<0.05), and similar to that of mDCs in both groups of subjects. Finally, pattern recognition receptors were also assessed in DCs. TLR2 expression was similar in all experimental conditions, despite showing a slight tendency to increase in PSAB-DCs in patients. CD14 was also similarly expressed in iDCs and mDCs, but mPSAB-DCs showed lower levels of CD14 expression in comparison to mDCs in control subjects and in comparison to iDCs in patients (p<0.05). DC-SIGN displayed a tendency to be downmodulated in PSAB-DCs, especially in controls, which was more marked after the addition of a maturation stimulus in mPSAB-DCs. Nonetheless, this marker showed a tendency to remain higher in PSAB-DCs than in mDCs in patients.

Cytokine secretion was also analysed in supernatants of DCs cocultures with PSAB-liposomes (**Figure 22**). Regarding the secretion of pro-inflammatory cytokines, IL-6 was released in low amounts in iDCs and after PSAB-liposomes phagocytosis. Nonetheless, the secretion of IL-6 by PSAB-DCs from patients with T1D was higher than in iDCs (p<0.05). As expected, its secretion increased after addition of a maturation stimulus in mPSAB-DCs and mDCs, and both control subjects and patients with T1D followed the same pattern (p<0.05, **Figure 22A**). TNF-α was determined in the four DCs culture conditions although both mPSAB-DCs and mDCs had received TNF-α as maturation stimuli in the CC added to the culture (**Figure 22B**). PSAB-DCs secreted TNF-α in similar levels to iDCs both in control subjects and patients, which were lower than those found in mPSAB-DCs and mDCs cultures

(p<0.05). As for the secretion of anti-inflammatory cytokines, PSAB-DCs from patients with T1D displayed a slight tendency to increase IL-10 secretion, although differences were not statistically significant (**Figure 22C**). Regarding the secretion of TGF-β1, PSAB-liposomes engulfment maintained a high profile of TGF-β1 secretion both in DCs from control subjects and patients (**Figure 22D**). Although statistical significance was not reached, mPSAB-DCs and mDCs tended to release lower levels of TGF-β1 when compared to iDCs and PSAB-DCs. IL-2, IL-17A and IFN-γ were not detected in any condition of the assay (data below the detection limit). IL-4 was not considered as it was used in culture media for DC differentiation.

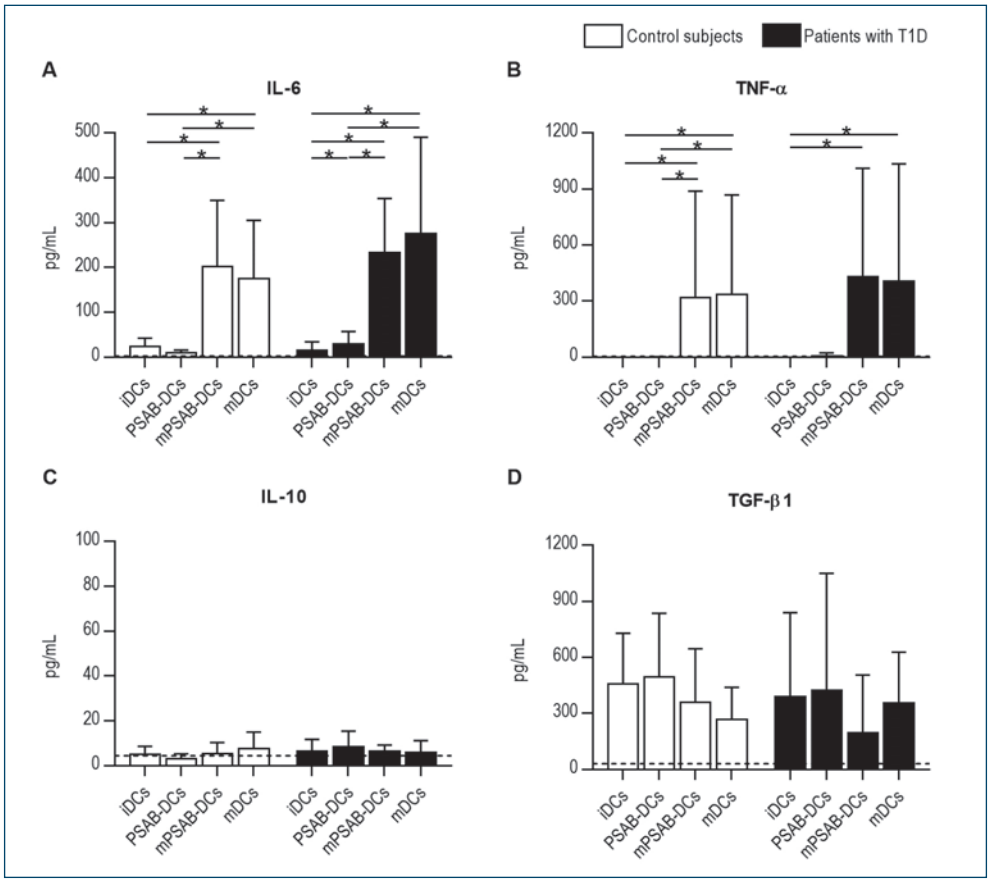


Figure 22. ***The uptake of PS-liposomes by DCs does not alter cytokine profile.*** **(A)** *Concentration (pg/mL) of IL-6,* **(B)** *TNF-α,* **(C)** *IL-10 and* **(D)** *TGF-β1 secreted by DCs obtained from control subjects (white bars, n≥3) and patients with T1D (black bars, n≥3). Bars represent iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), PSAB-DCs matured with CC (mPSAB-DCs), or mDCs (induced by CC), 24 hours after culture; discontinuous lines mark the limit of detection of the assay. Data presented as mean ± SD. Significant differences were found when comparing culture conditions in the same group of subjects (*p≤0.05, Wilcoxon test), and differences were not found when comparing the same culture condition between control subjects and patients with T1D. From Rodriguez-Fernandez* et al.*, 2018.*

15. Capture of PS-liposomes by adult DCs impairs their ability to stimulate autologous T cell proliferation

DCs, after being exposed to the different stimuli for 24 hours, were cocultured with autologous PBMCs for 6 days to assess their proliferation induction ability. As shown in **Figure 23**, DCs derived from patients with T1D and control subjects induced similar levels of autologous T cell proliferation. As expected, $CD4^+$ T cell proliferation (**Figure 23A**) induced by mDCs was higher than the proliferation induced by iDCs, but only reaching significance in patients ($p<0.0001$). Also, the levels of proliferation induced by PSAB-DCs were similar to those of iDCs in both groups, and lower from the levels induced by mDCs in patients with T1D ($p<0.01$). As for $CD8^+$ T cell proliferation (**Figure 23B**), that induced by mDCs was higher than that elicited by iDCs in control subjects ($p<0.05$), but not in patients. In the case of control subjects, the capture of PSAB-liposomes by DCs did not increase autologous $CD8^+$ T cell proliferation when compared to iDCs but, in the case of patients, it even induced a significant decrease of $CD8^+$ T cell proliferation ($p<0.01$). This effect was reverted after maturation in mPSAB-DCs and mDCs ($p<0.05$).

Regarding the cytokine production, PBMCs cocultured with PSAB-DCs displayed similar IL-6 and IFN-γ secretion to iDCs in both control subjects and patients with

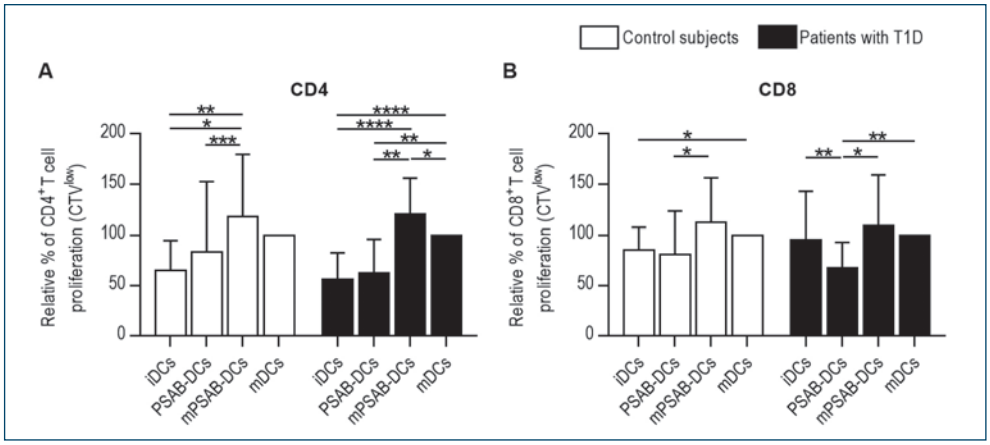


Figure 23. ***Phagocytosis of PSAB-liposomes affects the ability of DCs to induce autologous proliferation.*** **(A)** *Autologous proliferation of $CD3^+CD4^+$ T cell and* **(B)** *$CD3^+CD8^+$ T cell subsets induced by DCs obtained from control subjects (white bars, n=12) and patients with T1D (black bars, n≥12), after 6 days of coculture at 1:10 ratio (DCs:PBMCs). Bars represent iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), PSAB-DCs matured with CC (mPSAB-DCs), or mDCs (induced by CC). Data presented as mean ± SD of relative proliferation induction, this being the percentage of CTV^{low} cells in each coculture condition referred to that of their respective mDCs control. Significant differences were found when comparing culture conditions in the same group of subjects (*p≤0.05, **p<0.01, ***p<0.001, ****p<0.0001, Wilcoxon test), and differences were not found when comparing the same culture condition between control subjects and patients with T1D. From Rodriguez-Fernandez* et al., *2018.*

T1D, and showed a tendency to be lower than those of mDCs (**Figure 24A-B**). Also, although no significant differences were found, cocultures with patients' cells showed a trend for higher levels of IL-6 and IFN-γ secretion when compared to cocultures with control subject's cells, regardless of the DCs condition. IL-2, IL-4, IL-10, IL-17A and TNF-α were not detected in any condition of the assay (data below the detection limit).

16. Transcriptional changes in DCs from adult patients with T1D after PS-liposomes phagocytosis point to an immunoregulatory profile

Within the 34 adult patients with T1D recruited for the study of PS-liposomes' effect in adult DCs, we selected 8 specific patients (50% female) with a more stringent inclusion and exclusion criteria to participate in the RNA-seq analysis (see section 3.2). These criteria were applied in order to minimise the effect that long-term hyperglycaemia could have on genetic and epigenetic profiles. Further information on the subjects can be found in **Table 21**.

Thus, DCs were cocultured for 4 hours in basal conditions and with PSAB-liposomes to isolate RNA material. All RNA samples had an RNA integrity number (RINe) value >8.1, which was optimal for RNA-seq experiments. Further information on each RNA sample can be found in **Table 22**. For each patient, the ability of DCs to phago-

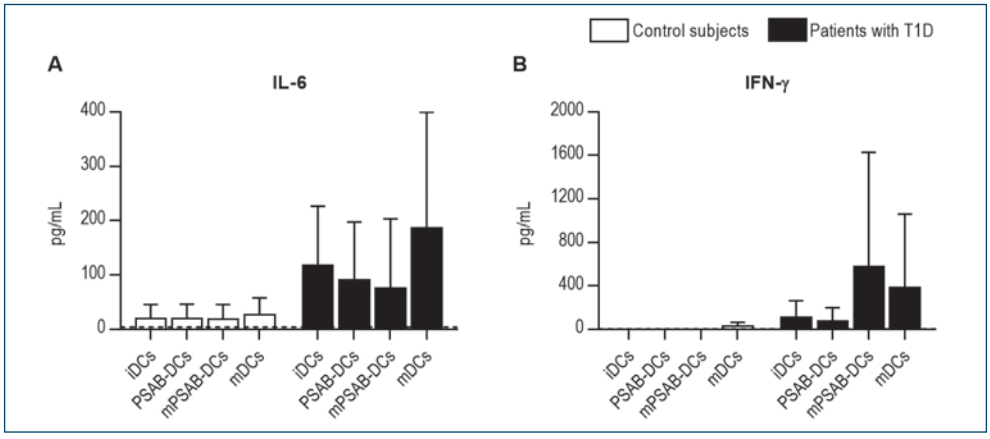


Figure 24. ***Cocultures of autologous PBMCs and DCs do not show alterations in pro-inflammatory cytokine production.*** **(A)** *IL-6 and* **(B)** *IFN-γ secretion assessed in supernatants of autologous proliferation cocultures at 1:10 ratio (DCs:PBMCs) for 6 days with cells from control subjects (white bars, n≥3) and patients with T1D (black bars, n≥3). Bars represent iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), PSAB-DCs matured with CC (mPSAB-DCs), or mDCs (induced by CC). Data presented as mean ± SD. No differences were found when comparing conditions in the same group of subjects, or when comparing the same culture condition between control subjects and patients with T1D. From Rodriguez-Fernandez* et al.*, 2018.*

Table 21. Data from the adult patients with T1D included in the RNA-seq analysis

Patient number	Gender	Age (years)	BMI (kg/m^2)	Age at T1D diagnosis (years)	Duration of T1D (years)	HbA1c (%)	HbA1c (mmol/mol)
1	Male	23	21.2	19	4	6.7	49.7
2	Female	28	24.4	23	5	6.5	47.5
3	Male	28	23.0	25	3	7.4	57.4
4	Female	33	21.4	33	0.5	5.9	41
5	Female	35	24.4	34	1	6.4	46.5
6	Female	32	18.6	31	0.5	5.9	41
7	Male	38	24.2	36	1	12.2	109.8
8	Male	21	23.0	16	5	7.9	62.8
Mean ± SD		29.75 ± 5.85	22.50 ± 2.00	27.13 ± 7.43	2.50 ± 1.98	7.36 ± 2.07	56.97 ± 22.65

Data presented as mean ± SD; *p*-value calculated from Mann-Whitney test. BMI: body mass index; HbA1c: glycated haemoglobin. From Rodriguez-Fernandez *et al.*, 2018.

Table 22. Features of iDCs and PSAB-DCs samples used in the RNA-seq experiment

Patient number	Condition	Cell number (×10^6)	RNA quantity (µg)	RINe	28S/18S (area)	PSOG488-liposomes phagocytosis (%)
1	iDCs PSAB-DCs	4.42 5.05	12.7 20.9	8.1 9	1.9 2	60.2
2	iDCs PSAB-DCs	1.58 1.88	6.3 7.2	9.4 9.5	2.2 1.9	78.5
3	iDCs PSAB-DCs	2.03 2.83	3.5 5.5	8.4 8.3	1.5 1.5	83.2
4	iDCs PSAB-DCs	1.44 1.77	4 4.4	8.7 8.3	1.9 1.3	72.9
5	iDCs PSAB-DCs	2.16 2	4.4 8.4	8.8 8.4	1.6 1.8	90
6	iDCs PSAB-DCs	2.35 2.17	3.3 5.1	9.5 9.4	2.2 2.4	82
7	iDCs PSAB-DCs	1.45 1.39	3.2 3.3	9.5 9.6	1.9 1.9	66.4
8	iDCs PSAB-DCs	1.66 1.52	3.5 5.7	9.6 9.5	2.4 2.6	57.8
Mean ± SD		2.23 ± 1.06	6.33 ± 4.60	9.00 ± 0.56	1.94 ± 0.36	73.88 ± 11.57

RINe: RNA integrity number; iDCs: immature dendritic cells; PSAB-DCs: dendritic cells cocultured with PSAB-liposomes.

cyte PS-liposomes was verified by flow cytometry using PSOG488-liposomes, and all samples achieved an average of 73.88 ± 11.57% (mean ± SD) fluorescent signal.

After library preparation and sequencing, bioinformatics analysis of the RNA-seq experiment allowed for the allocation of gene sequences to specific genes and determination of their differential expression in iDCs and PSAB-DCs. Experimental data have been uploaded into the public repository European Nucleotide Archive (www.ebi.ac.uk/ena; accession number: PRJEB222240). Further analysis revealed that only 233 of 22,711 genes sequenced were differentially expressed between iDCs and PSAB-DCs, considering a *p*-value<0.0013, adjusted *p*-value<0.1254, and Log_2 of fold change >0.05 or <–0.05. Of these 233 genes, 203 (87.12%) were found downregulated and the remaining 30 genes (12.88%) were found upregulated. Also, out of the 233 genes, 224 corresponded to protein-coding genes. Despite the heterogenous transcriptomics of DCs in basal conditions (iDCs) from the 8 patients, gene expression was markedly altered towards a similar profile after PSAB-liposomes phagocytosis (**Supplementary Figure 8**).

To define altered signalling pathways in DCs after phagocytosis of PSAB-liposomes, information on each gene was collected through different databases (see section 5.8.2 of Material and Methods). Furthermore, these DEGs were classified in broad functional categories (**Supplementary Table 1**), which are outlined in **Table 23**.

Several categories of DEGs and molecules were related to DC function. In fact, the DEGs found were mainly related to metabolism, gene expression, immunoregulation, signal transduction, molecule transport, post-translational protein modification, cytokine signalling, cell cycle, vesicle-mediated processes, DNA replication and repair, antigen processing and presentation, apoptosis and cytoskeleton organisation. Since PS-liposomes therapy has immunotherapeutic potential, the DEGs involved in tolerance promoting pathways were analysed thoroughly. DEGs linked to the immune system were primarily downmodulated and involved in antigen processing and presentation (*KBTBD6, BTK, CDC23, UBE2E3, CD1D*), regulation of the immune response (*DAPP1, GIMAP4, SLAMF6*) and cytokine signalling relevant in the interaction between T cells and DCs (*SOCS2, TNFRSF11A*). However, although very few genes were upregulated after PSAB-liposomes phagocytosis in DCs, these were involved in the prevention of DC maturation (*TNFSF14, TNFAIP3, VEGFA, SHB, LAIR1, NFKBIA*). Also, in the vesicle-mediated processes category, the genes involved in intracellular trafficking were found downmodulated (*GOLPH3L, SEC22C, RAB32, KIF20B*), whereas the two hyperexpressed genes (*CYTH4, LDLR*) are known to participate in the binding of lipids and internalisation of lipid particles. In this sense, in the metabolism category, the *HPGD* gene, which reportedly contributes to prostaglandin inactivation, was found downmodulated in PSAB-DCs. Finally, genes

Table 23. Summary of DEGs in DCs from patients with T1D after PSAB-liposomes phagocytosis

Category	Number of DEGs	*p*-value	Representative downregulated genes	Representative upregulated genes
Adhesion	3	≤0.001076	*SCYL3, MEGF9*	*IGSF9*
Antigen processing and presentation	7	≤0.001024	*KBTBD6, BTK, CDC23, UBE2E3, CD1D, CUL3, KIF11*	
Apoptosis	6	≤0.000593	*BLCAP, PMP22, LMNB1, CASP3, DCAF7, BCL2L1*	
Cell cycle	9	≤0.001162	*CSRP2BP, BUB1, MCPH1, CDK13, PCNA, MCM4, SMC2, NCAPG2, AURKA*	
Cytokine signalling	9	≤0.001191	*TRIM5, SOCS2, STX3, TNFRSF11A, NUP160*	*TNFSF14, VEGFA, TNF, IFNLR1*
Cytoskeleton organisation	6	≤0.000897	*MAPRE2, RMDN1, CKAP2, MDM1, RCSD1, CDC42SE1*	
DNA replication and repair	8	≤0.000995	*WRNIP1, PAXIP1, MSH2, RAD51C, DCLRE1A, ALKBH1, PARG, MLH1*	
Gene expression	36	≤0.001293	*ZNF436, MYB, ZFP36L2, MIER3, ZBTB5, HHEX, GTF2B, DYRK2, NFIA, ZBTB39*	
Immunoregulation	25	≤0.001146	*GIMAP4, SLAMF6, DAPP1, MEF2C, BST1, PROS1, MNDA*	*TNFAIP3, PLAUR, NFKBIA, LAIR1*
Metabolism	43	≤0.001225	*C9orf64, HPGD, TIMMDC1, ICK, DDO, DCTD, CDYL2, GLRX, TPK1*	*MFSD2A*
Molecule transport	14	≤0.001252	*ERLIN1, SLC10A7, UNC50, ATP10D, SLC40A1, CLCN3, STIM2*	*SLC43A3, SLCO4A1, CLCN6*
Post-translational protein modification	13	≤0.001056	*FBXO36, NSMCE4A, VWA5A, FBXO25, DCAF12, CBX4, RMND5A, LNX2, BTBD3*	*PPME1*
Signal transduction	18	≤0.001298	*SNN, SKI, PAQR8, UBFD1, N4BP1, FZD5, NET1, ZBED3, FRAT2*	*SHB*
Vesicle-mediated processes	8	≤0.001208	*GOLPH3L, SEC22C, RAB32, EHBP1, KIF20B, SNX18*	*CYTH4, LDLR*

DEGs: differentially expressed genes. From Rodriguez-Fernandez *et al.*, 2018.

related to apoptosis were downregulated in DCs after liposome uptake (*BLCAP*, *PMP22*, *CASP3*, *BCL2L1*).

Validation of the RNA-seq experiment results was performed by qRT-PCR. The expression of the eight selected gene targets, namely *CYTH4*, *GIMAP4*, *HPGD*, *NFKBIA*, *PLAUR*, *TNFAIP3*, *TNFSF14*, and *VEGFA* genes, was tested in iDCs and PSAB-DCs of patients with T1D and confirmed the RNA-seq findings (**Figure 25**). The same assay was performed with iDCs and PSAB-DCs from control subjects, and they showed the

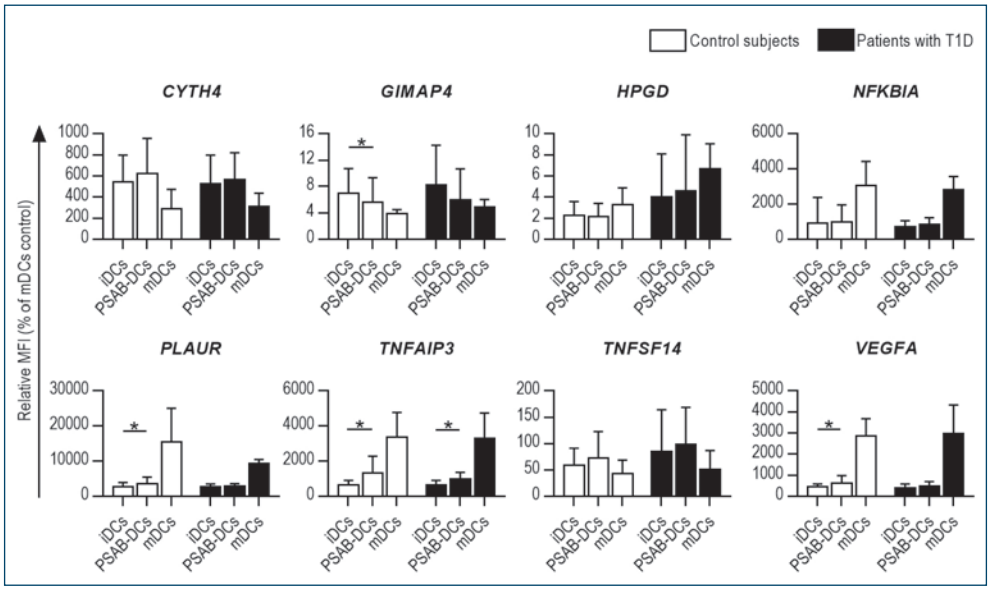


Figure 25. ***Quantitative RT-PCR validates the RNA-seq results.*** *Relative gene expression of 8 selected targets in iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), and mDCs, analysed by qRT-PCR. Gene expression signals were normalised to the housekeeping* GAPDH *gene. Bars show the mean ± SD of gene expression in control subjects (white bars, n=3) and patients with T1D (black bars, n=4). Statistically significant differences were found when comparing conditions in the same group of subjects (*p<0.05, Wilcoxon test), *and differences were not found when comparing the same culture condition between control subjects and patients with T1D. From Rodriguez-Fernandez* et al., *2018.*

same pattern as the patients with T1D did. Finally, in order to know how a maturation stimulus affected the expression of these genes, mDCs condition in control subjects and patients with T1D was also analysed by qRT-PCR. In mDCs, we observed an apparently different gene expression pattern when compared to PSAB-DCs and iDCs. Genes upregulated by PS-liposomes, such as *CYTH4* and *TNFSF14*, tended to be downregulated in mDCs; other genes showed a trend for higher expression in mDCs (*NFKBIA*, *PLAUR*, *TNFAIP3*, *GIMAP4*, *VEGFA*, and *HPGD*) in comparison to the other conditions.

17. Paediatric patients with T1D at onset and with established disease differ in age, BMI and HbA1c levels

With the aim to assess the changes that DCs from paediatric patients undergo after PS-liposomes phagocytosis, 11 control subjects (73% female), 14 patients with T1D at onset (64% female) and 20 patients with established disease (60% female) from the Germans Trias i Pujol Hospital were recruited to donate a 10 mL blood sample. All subjects met the inclusion and exclusion criteria and gave informed consent to participate in the study. Their data is summarised in **Table 24**. Statistically significant

Table 24. Data from the paediatric patients with T1D and paediatric control subjects recruited for the study

Parameter	Paediatric control subjects	Paediatric patients with T1D at onset	Paediatric patients with established T1D
N	11	14	20
Gender	8/11 (73%) Female 3/11 (27%) Male	9/14 (64%) Female 5/14 (36%) Male	12/20 (60%) Female 8/20 (40%) Male
Age (years)	9.56 ± 1.69	9.74 ± 3.55	12.41 ± 4.01*
BMI (kg/m^2)	17.50 ± 2.61	16.57 ± 3.08	20.31 ± 3.70*/$^{++}$
Age at T1D diagnosis (years)	NA	9.74 ± 3.55	8.85 ± 3.63
Duration of T1D (years)	NA	0.00 ± 0.00	3.56 ± 2.97^{++++} (range 0.5-11.7 years)
HbA1c (%)	NP	12.54 ± 1.91	7.36 ± 1.91^{++++}
HbA1c (mmol/mol)	NP	113.51 ± 20.93	58.11 ± 16.31^{++++}
Fasting C-peptide (ng/mL)	NP	0.46 ± 0.28	<0.1 in 14/20 patients (70%) 0.4 ± 0.32
Stimulated C-peptide (ng/mL)	NP	0.88 ± 0.58	NP
Insulin dose (IU/kg/day)	NA	0.85 ± 0.22	0.87 ± 0.18

Data presented as mean ± SD; *p*-value calculated from Mann-Whitney test (*p<0.05 when comparing either group of patients to the control group; ^{++}p<0.01, ^{++++}p<0.0001 when comparing paediatric patients at onset and with established disease). T1D: type 1 diabetes; BMI: body mass index; HbA1c: glycated haemoglobin; NA: not applicable; NP: not performed.

differences were found when comparing the age between patients with established disease and control subjects (p<0.05), although significance was not reached when comparing the age of patients with established disease with that of the onset group (p=0.06). Also, the BMI of patients with established T1D was higher than that of control subjects (p<0.05) and that of patients at onset (p<0.01). As expected, differences were found in the duration of the disease and in HbA1c levels between patients at onset and patients with established disease (p<0.0001).

18. DC generation is comparable in paediatric patients with T1D and control subjects

Monocytes were magnetically isolated from whole blood obtained from paediatric subjects to be differentiated into DCs. The absolute number of CD14$^+$ isolated cells, the purity of CD14$^+$ cells and viability, and the purity of CD11c$^+$ cells at days 1, 4 and 6 of cell culture —as means of monitoring their differentiation efficiency into DCs— are shown in **Table 25**. When comparing the groups, the percentage of CD14$^+$ cells in the isolated fraction of paediatric patients with established T1D was found significantly lower than that of control subjects. However, the three groups displayed similar dynamics of CD11c marker acquisition and DC differentiation.

Table 25. Data from monocyte's isolation and differentiation into DCs of paediatric subjects

Parameter	Paediatric control subjects	Paediatric patients with T1D at onset	Paediatric patients with established T1D
Isolated CD14$^+$ cells (day 1, × 10^6 cells)	2.60 ± 0.84	2.83 ± 1.46	2.54 ± 1.05
CD14 purity (day 1, %)	94.95 ± 3.86	87.21 ± 12.31	86.98 ± 9.20*
Viability (day 1, %)	96.13 ± 2.34	96.29 ± 3.33	97.20 ± 1.83
CD11c purity (day 1, %)	4.23 ± 3.24	4.56 ± 2.67	5.41 ± 4.87
Differentiation efficiency (CD11c purity, day 4, %)	63.50 ± 18.59	61.46 ± 25.27	72.37 ± 25.21
Differentiation efficiency (CD11c purity, day 6, %)	90.17 ± 6.06	88.31 ± 9.19	87.22 ± 12.79

Data presented as mean ± SD; *p*-value calculated from Mann-Whitney test (*p<0.05 comparing patients with established T1D with the control group). Isolated CD14$^+$ cells: absolute number of CD14$^+$ cells obtained after isolation. CD14 purity: percentage of CD14$^+$ cells in the isolated fraction. Viability: percentage of Annexin V$^-$ 7-AAD$^-$ cells of isolated CD14$^+$ cells. CD11c purity: percentage of CD11c$^+$ cells in the isolated fraction. Differentiation efficiency: percentage of CD11c$^+$ cells.

19. DCs from paediatric patients with established T1D show altered PS-liposomes phagocytic capacity in correlation with disease evolution time

In order to discard any detrimental effects of PS-liposomes on DCs viability, this was assessed by flow cytometry after 24 hours of coculture (**Figure 26**). DCs from paediatric control subjects and paediatric patients with T1D at onset and with established disease showed a viability higher than 87% in each condition, thus meaning that 1 mM PS-liposomes is not toxic to DCs obtained from paediatric subjects.

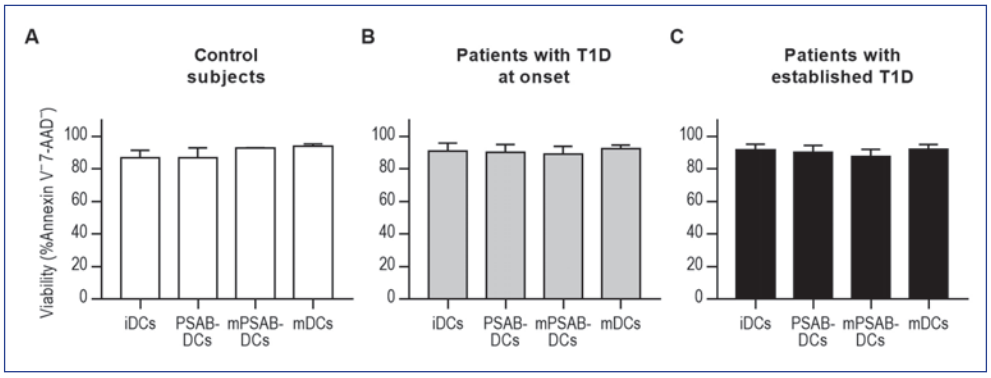


Figure 26. ***PS-liposomes uptake preserves high viability in DCs from paediatric subjects.*** **(A)** *Viability of DCs from control subjects (white bars, n≥2),* **(B)** *patients with T1D at onset (grey bars, n≥3) and* **(C)** *patients with established T1D (black bars, n≥9) assessed by annexin V and 7-AAD staining. Bars represent iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), PSAB-DCs matured with CC (mPSAB-DCs), or mDCs (induced by CC), after 24 hours of coculture.*

Furthermore, a time course experiment was performed at 37 °C with DCs from the different paediatric groups to assess PS-liposomes phagocytosis kinetics (**Figure 27**). A control of capture at 4 °C was performed for each sample to confirm that paediatric DCs could only phagocyte PSOG488-liposomes at 37 °C (data not shown), as it was the case in adult DCs (**Figure 19**). As shown in **Figure 27A**, the capture of PSOG488-

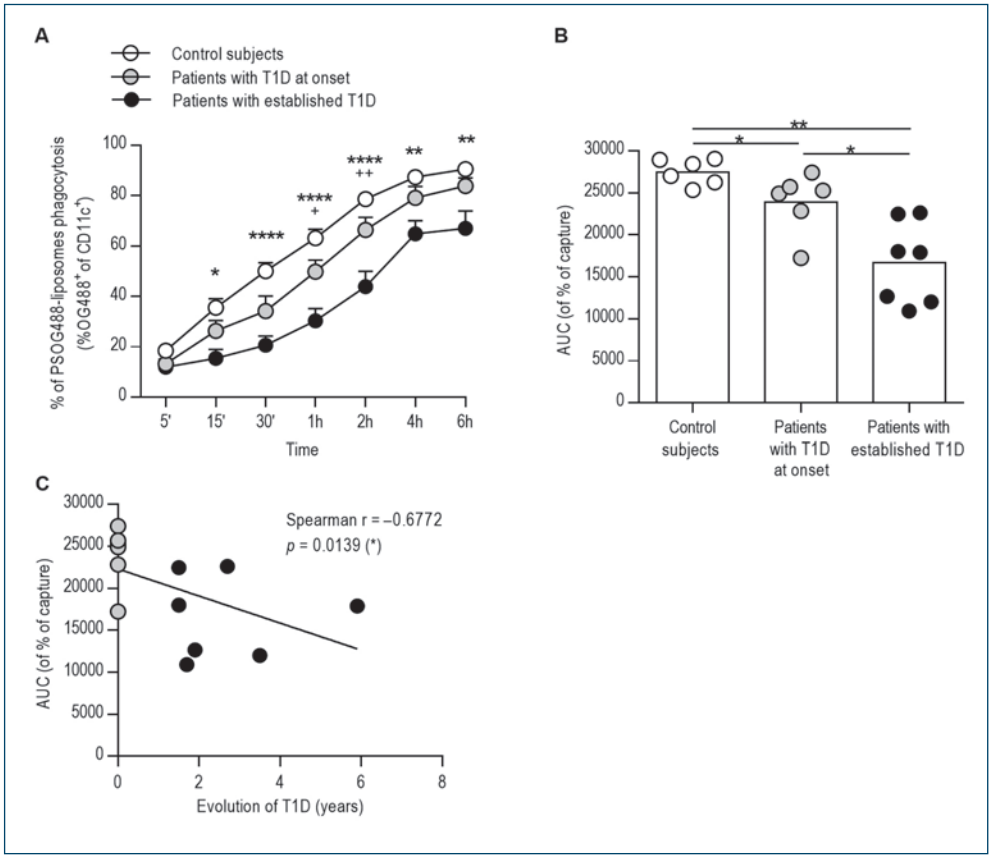


Figure 27. ***PS-liposomes are captured less efficiently by DCs from paediatric patients with T1D in correlation with the time of disease evolution.*** **(A)** *Time course of the capture of PSOG488-liposomes by DCs obtained from control subjects (white symbols, n=6), paediatric patients with T1D at onset (grey symbols, n=6) and with established T1D (black symbols, n=7) at 37 °C. Results are mean ± SEM. Comparisons between groups showed significant differences (*p<0.05, **p<0.01, ****p<0.0001 when comparing control subjects with patients with established T1D; +p<0.05, ++p<0.01 when comparing patients at onset with patients with established disease, Two-way ANOVA).* **(B)** *AUC of phagocytosis kinetics curves. White symbols represent control subjects (n=6); grey symbols, paediatric patients with T1D at onset (n=6) and black symbols, patients with established T1D (n=7). Differences were found between groups (*p<0.05, **p<0.01, Mann-Whitney test).* **(C)** *Correlation between AUC values of phagocytosis kinetics curves and evolution of T1D (grey symbols are patients at onset, n=6; black symbols are patients with established T1D, n=7). A significant correlation was found between the two parameters (Spearman's r = –0.6772,* p*<0.05, Spearman's correlation analysis).*

liposomes was significantly lower in DCs from patients with established T1D when compared to that of DCs from control subjects, starting at 15 minutes of coculture (p<0.05). Also, the capture PSOG488-liposomes by DCs from patients with established disease was decreased in comparison to the capture displayed by DCs from patients at onset, but only at the 1 hour and 2 hours' time points. Moreover, PS-liposomes uptake kinetics showed a tendency to be lower in DCs from patients at onset when compared to that of control subjects, but differences were not statistically significant. To further dissect this finding, we compared the AUC obtained from the PSOG488-liposomes capture curves of the three groups (**Figure 27B**). Indeed, the AUC from the control subjects' group was higher than the AUCs obtained from patients at onset (p<0.05) and patients with established disease (p<0.01), and patients at onset also displayed higher AUC than that of patients with established T1D (p<0.05). When analysing the correlation between these AUCs values and the time of evolution of T1D, we discovered that the AUC of capture kinetics was negatively correlated with that time, and that the correlation was statistically significant (Spearman r = –0.6772, p=0.0139, **Figure 27C**). The analysis of correlation of the AUC values with other clinical parameters (age, BMI, age at T1D diagnosis, HbA1c levels, fasting C-peptide and insulin dose) was also performed, but no significant correlations were observed (**Supplementary Table 2**).

The comparison of the capture kinetics of PSOG488-liposomes and PCOG488-liposomes was also carried out in selected subjects (1 control subject and 2 paediatric patients at onset), and the results are shown in **Supplementary Figure 9**. Although no significant differences were found, DCs from both groups exhibited a trend for a more efficient and quicker phagocytosis kinetics when cocultured with PSOG488-liposomes than when exposed to PCOG488-liposomes (**Supplementary Figure 9A**). When converting these data into AUCs, the AUC of phagocytosis kinetics of PCOG488-liposomes appeared lower than that of PSOG488-liposomes in DCs from both control subjects and patients with T1D at onset (**Supplementary Figure 9B**).

20. The expression of phagocytosis-related genes is altered in DCs from paediatric patients with T1D

When discovering that the phagocytosis kinetics negatively correlated with the time of disease evolution, we decided to study the changes in the expression of certain genes involved in phagocytosis, efferocytosis and lipid metabolism after 4 hours of PSAB-liposomes coculture (**Figure 28**). Namely, CD36 and CD68 are two scavenger receptors that recognise PS and other ligands, MerTK is a TAM receptor that indirectly recognizes PS via proteinS or Gas6 protein, MFG-E8 is a bridging molecule be-

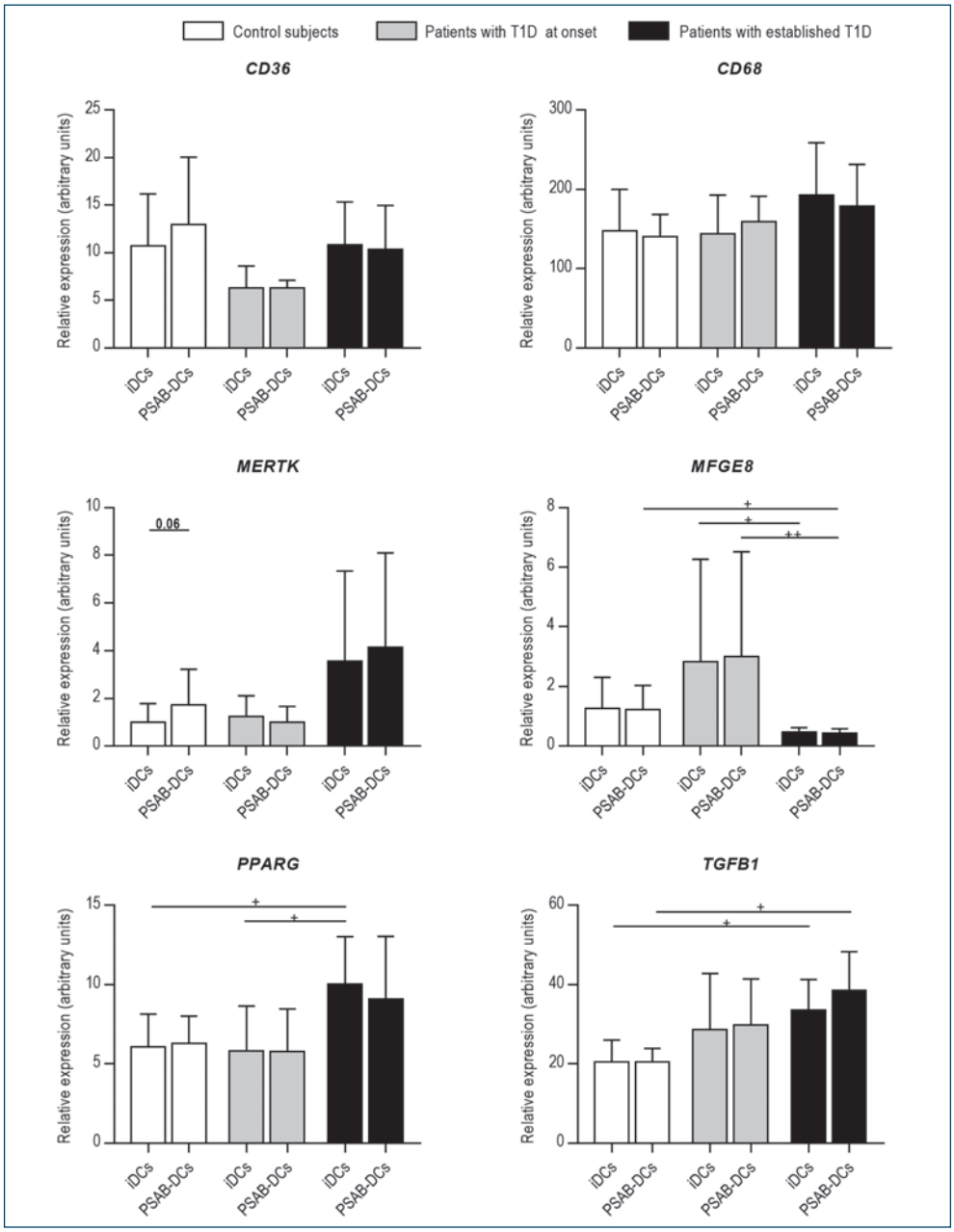


Figure 28. ***The expression of genes related to phagocytosis, lipid metabolism and efferocytosis in DCs differs between groups.*** *Expression of* CD36, CD68, MERTK, MFGE8, PPARG *and* TGFB1 *genes in iDCs and PSAB-DCs after 4 hours of being cocultured with PSAB-liposomes, analysed by qRT-PCR. Gene expression signals were normalised to* GAPDH *expression. White bars represent control subjects (n=5), grey bars represent patients with T1D at onset (n=5) and black bars represent patients with established T1D (n=6); bars show the mean ± SD. Significant differences were not found when comparing culture conditions in the same group (Wilcoxon test), but were found when comparing the same culture condition between the three groups (*$^{+}$p*<0.05,* $^{++}$p*<0.01, Mann-Whitney test).*

tween PS and αvβ3/β5 integrins (see **Figure 1A**), PPARγ (*PPARG* gene) is involved in lipid metabolism and regulation of inflammation, and TGF-β1 (*TGFB1* gene) is reportedly secreted by DCs upon efferocytosis to promote self-tolerance (Kimani *et al.*, 2014; Szatmari *et al.*, 2006; Vives-Pi *et al.*, 2015). First, *CD36* gene displayed a tendency to be expressed at lower levels in DCs from patients at onset than in control subjects or patients with established disease. Interestingly, DCs from control subjects showed a trend to upregulate *CD36* expression after phagocytosis of PSAB-liposomes, whereas in patients, it remained unaltered or showed a slight trend for downregulation. As for *CD68*, DCs from patients with established disease tended towards a higher expression than the other groups. Regarding the effect of PSAB-liposomes, *CD68* showed a trend to be downregulated in DCs from control subjects and patients with established disease, but upregulated in patients at onset. Regarding *MERTK*, DCs from patients with established disease had a clear tendency for a greater expression than the other groups. When exposed to PSAB-liposomes, DCs from control subjects and patients with established disease had a trend to upregulate *MERTK* expression, while it was reversed in patients at onset. Considering *MFGE8*, DCs from patients with established disease showed a lower expression than their counterparts from patients at onset and control subjects ($p<0.05$). The coculture with PSAB-liposomes did not induce any significant changes in its expression. As for *PPARG*, DCs from patients with established disease showed a greater expression of the gene than DCs from control subjects and patients at onset ($p<0.05$). Regarding the effect of PSAB-liposomes, PSAB-DCs from patients with established disease showed a trend for downregulation of *PPARG*, whereas it remained mostly unaltered in the other groups. Finally, the expression of the *TGFB1* gene was higher in DCs from patients with established disease than in controls, and DCs from patients at onset showed the same trend. Interestingly, when exposed to PSAB-liposomes, only DCs from patients with T1D displayed a trend for *TGFB1* gene upregulation, and this upregulation was most notable in patients with established disease.

21. PS-liposomes phagocytosis alters gene expression in DCs from paediatric patients pointing to immunoregulation

To further dissect the tolerogenic potential of PS-liposomes phagocytosis in DCs obtained from paediatric subjects, we studied the change in expression of genes involved in tolerogenic signalling pathways that were discovered upregulated in the adult RNA-seq, namely the *TNFAIP3*, *TNFSF14*, *VEGFA* and *LAIR1* genes (**Figure 29**). First, the levels of expression of *TNFAIP3* showed a tendency to be higher in DCs from patients, both at onset and with established disease, than in control subjects. Also, the uptake of PSAB-liposomes showed a trend to induce an upregulation

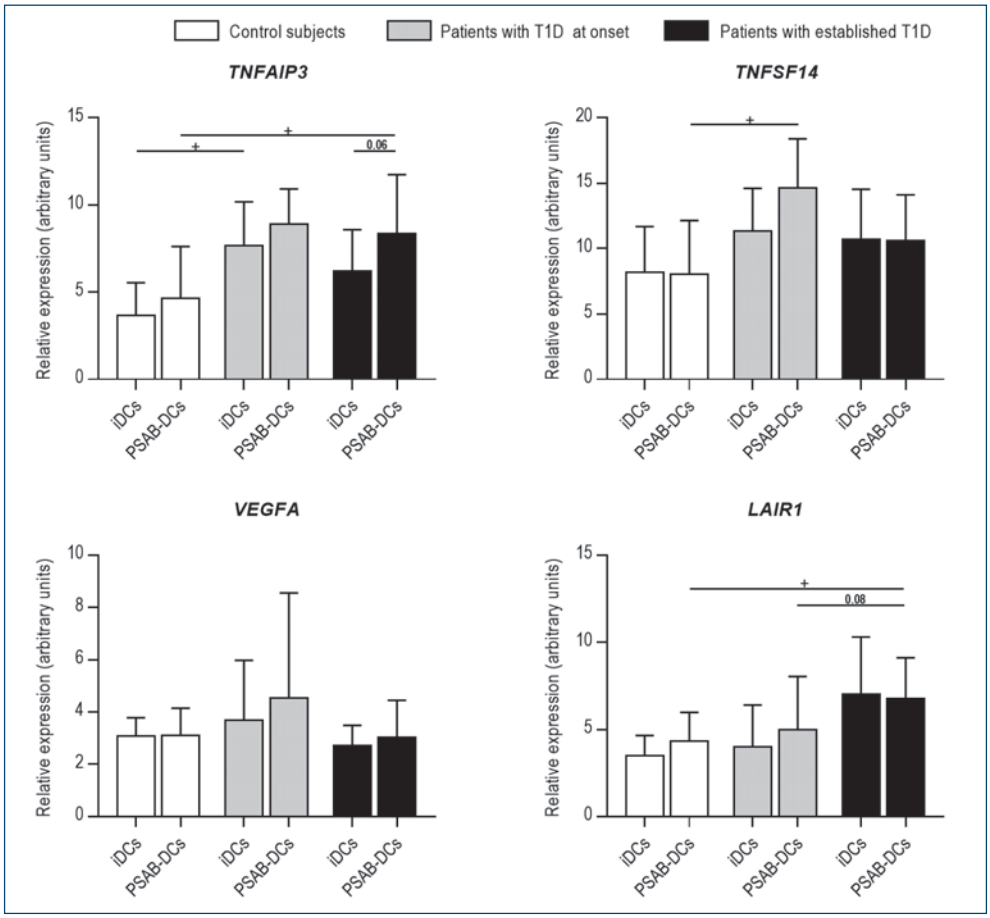


Figure 29. ***The expression of genes involved in tolerogenic pathways is different between the studied groups.*** *Expression of* TNFAIP3, TNFSF14, VEGFA *and* LAIR1 *genes in iDCs and PSAB-DCs after 4 hours of coculture with PSAB-liposomes, analysed by qRT-PCR. Gene expression signals were normalised to* GAPDH *expression. White bars represent control subjects (n=5), grey bars represent patients with T1D at onset (n=5) and black bars represent patients with established T1D (n=6); bars show the mean ± SD. Significant differences were not found when comparing culture conditions in the same group (Wilcoxon test), but were found when comparing the same culture condition between the three groups (*$^{+}$p*<0.05,* $^{++}$p*<0.01, Mann-Whitney test).*

of *TNFAIP3* expression in the three groups. As for *TNFSF14*, DCs from patients at onset displayed a tendency towards the highest expression, whereas DCs from patients with established disease had a trend for intermediate levels. Only PSAB-DCs from patients at onset displayed a tendency to upregulate *TNFSF14* expression after PSAB-liposomes phagocytosis, but the expression of this gene remained unaltered in PSAB-DCs from control subjects and patients with established disease. As for *VEGFA* gene, DCs from patients at onset had a trend for the highest expression. Also, they showed a tendency to upregulate *VEGFA* gene expression after PSAB-

liposomes phagocytosis, and PSAB-DCs from patients with established disease showed a similar trend. Finally, *LAIR1* gene expression was the highest in DCs from patients with established disease ($p<0.05$). After the capture of PSAB-liposomes, PSAB-DCs from control subjects and patients at onset showed a trend for upregulation of *LAIR1* expression, while in PSAB-DCs from patients with established disease, it displayed a slight tendency for downregulation.

22. PS-liposomes uptake drives a tolerogenic phenotype in DCs from paediatric patients with T1D

To determine whether the acquisition of a tolerogenic phenotype by DCs after PSAB-liposomes phagocytosis was also affected by the progression of the disease, DCs from paediatric patients with T1D at onset and with established disease were cultured for 24 hours in the different conditions. First, we studied the changes in expression of PS receptors CD36, TIM-4 and integrin αvβ5 (**Figure 30**). Regarding CD36, iDCs from patients at onset displayed a greater expression of this marker when compared to mPSAB-DCs ($p<0.05$). Even though statistical significance was not reached, PSAB-DCs showed a trend towards a higher expression of CD36 than mPSAB-DCs and mDCs. The expression of CD36 in DCs obtained from patients with established disease displayed the same pattern. As for TIM-4, iDCs and PSAB-DCs from patients at onset had a greater expression than mPSAB-DCs, and the same trend was observed when compared to mDCs. In patients with established T1D,

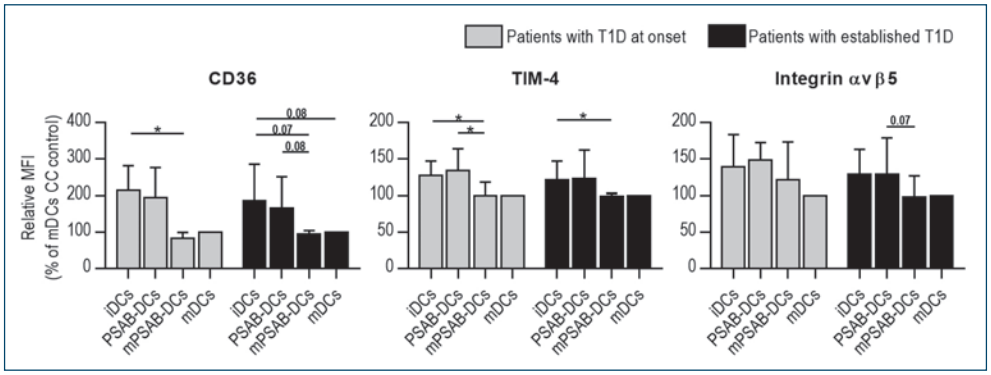


Figure 30. ***The expression of PS receptors is altered depending on the stimuli added to the culture.*** *Membrane expression of CD36, TIM-4 and integrin αvβ5 in DCs obtained from paediatric patients with T1D at onset (grey bars, n=3) and patients with established T1D (black bars, n≥4). Bars represent iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), PSAB-DCs matured with CC (mPSAB-DCs), or mDCs (induced by CC). Data presented as mean ± SD of relative MFI, this being MFI of each culture condition referred to their respective mDCs control. Significant differences were found when comparing culture conditions in the same group of subjects (*p≤0.05, Wilcoxon test), but not when comparing the same culture condition between patients at onset and patients with established disease.*

iDCs also showed higher levels of TIM-4 than mPSAB-DCs and, overall, its expression followed the pattern displayed by patients at onset. Finally, the expression of integrin αvβ5 did not show differences between conditions, although PSAB-DCs from patients with established disease displayed a trend to have higher levels of the marker than mPSAB-DCs (p=0.07).

Then, we also analysed the expression of different markers related to immunological functions, these being HLA-ABC and HLA-DR (antigen-presenting molecules), CD54 (adhesion molecule), CD86 and CD40 (costimulatory molecules), CD25 (activation molecules), CCR7, CCR2 and CXCR4 (chemokine receptors) and TLR2, CD14 and DC-SIGN (pattern recognition receptors). The results are shown in **Figure 31**. Regarding antigen-presenting molecules, treatment with PSAB-liposomes (PSAB-DCs and mPSAB-DCs) showed a trend to decrease the expression of HLA-ABC and HLA-DR in comparison to iDCs and mDCs, and this pattern was displayed by DCs obtained from patients with T1D at onset and with established disease. As for CD54, iDCs displayed the lowest expression in comparison to the other conditions in patients with established T1D (p<0.05), and PSAB-DCs showed a higher expression of this marker than iDCs (p<0.05), but lower than mPSAB-DCs and mDCs (p<0.05). In patients at onset, statistical significance was not reached, but the expression of CD54 followed the same pattern. As for the expression of costimulatory molecules CD86 and CD40, PSAB-DCs and mPSAB-DCs exhibited a significant decrease in their expression or a trend to have lower expression when compared to iDCs and mDCs, both in patients at onset and in patients with established T1D. By contrast, neither CD25 nor CCR7 displayed any changes of expression in any of the culture conditions analysed. As for chemokine receptor CCR2, significant differences were only found in DCs obtained from patients with established disease; therefore, iDCs and PSAB-DCs showed lower levels of expression of this marker than DCs cultured with maturation stimuli (mPSAB-DCs and mDCs, p<0.05). Regarding CXCR4, immature conditions of DCs (iDCs and PSAB-DCs) also showed a lower expression than mPSAB-DCs and mDCs in patients with established T1D (p<0.01), and the same trend was observed in DCs obtained from patients at onset. Regarding TLR2 expression, iDCs and PSAB-DCs from patients at onset displayed a greater expression than mDCs (p<0.05), and the same tendency was observed when comparing PSAB-DCs with mPSAB-DCs (p=0.06). No differences in TLR2 expression, however, were found when comparing conditions in DCs from patients with established disease. As for CD14, iDCs from patients with established T1D showed a greater expression than PSAB-DCs (p<0.05) and the same tendency when compared to mDCs (p=0.06). Also, PSAB-DCs displayed a higher expression of CD14 than mPSAB-DCs in this group (p<0.05). The expression of CD14 in the different conditions of DCs obtained from patients at onset exhibited the same pattern. Finally, DC-SIGN was expressed

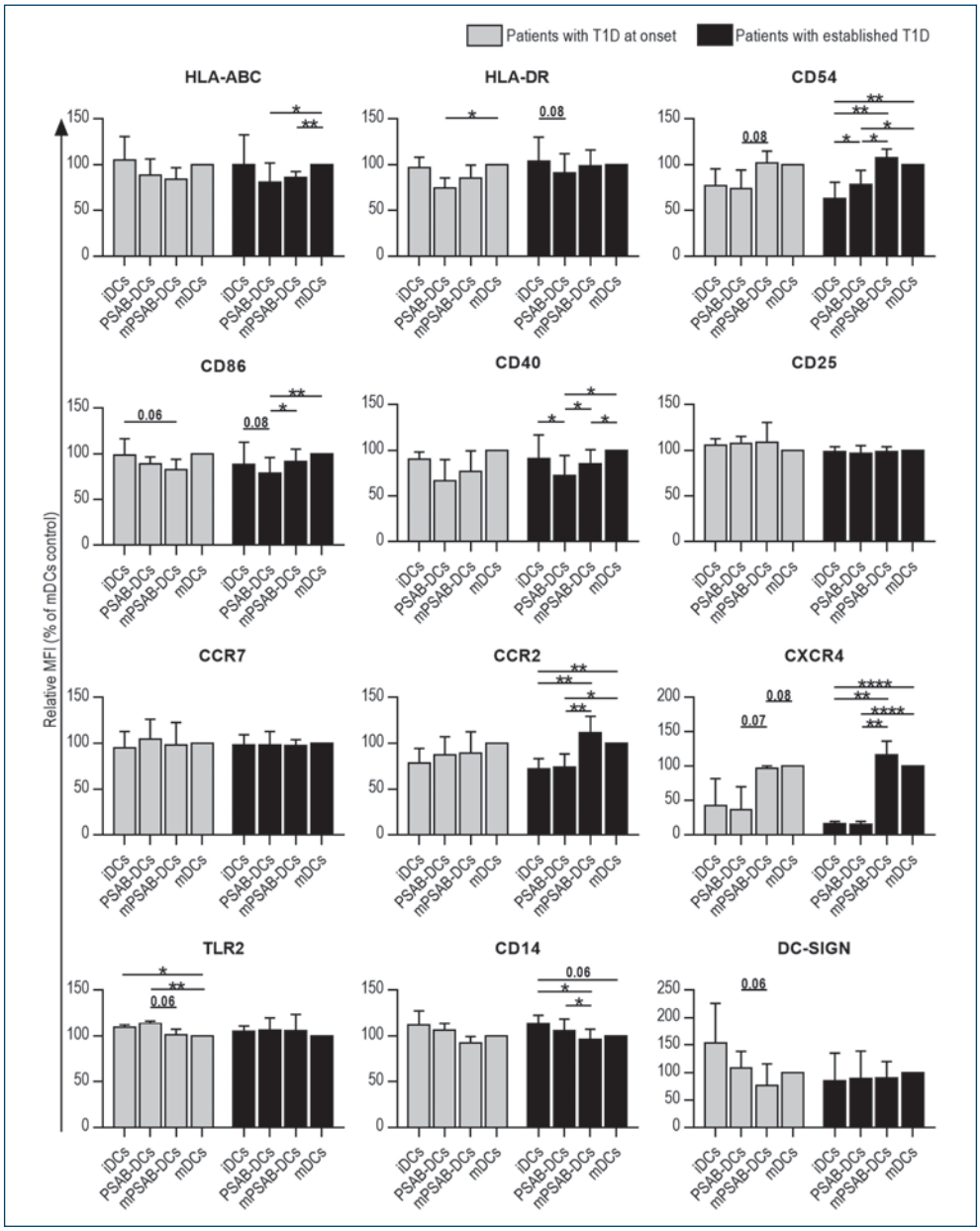


Figure 31. ***The expression of immune-related molecules changes in DCs after engulfment of PS-liposomes.*** *Membrane expression of HLA-ABC, HLA-DR, CD54, CD86, CD40, CD25, CCR7, CCR2, CXCR4, TLR2, CD14 and DC-SIGN in DCs obtained from paediatric patients with T1D at onset (grey bars, n=3) and patients with established T1D (black bars, n≥4). Bars represent iDCs, iDCs after the capture of PSAB-liposomes (PSAB-DCs), PSAB-DCs matured with CC (mPSAB-DCs), or mDCs (induced by CC). Data presented as mean ± SD of relative MFI, this being MFI of each culture condition referred to their respective mDCs control. Significant differences were found when comparing culture conditions in the same group of subjects (*p≤0.05, **p<0.01, ****p<0.0001, Wilcoxon test), but not when comparing the same culture condition between patients at onset and patients with established disease.*

similarly in all conditions of DCs, although a trend for downmodulation of its expression was observed in PSAB-DCs and mPSAB-DCs from patients at onset.

23. The percentage and number of DCs subsets from peripheral blood is altered in the 1st year of progression in paediatric patients with T1D

Since we had discovered a negative correlation between the progression of T1D and the ability of monocyte-derived DCs to capture PS-liposomes, we wondered if we could find alterations in numbers and percentages of peripheral blood DCs from children at different stages of the disease, which could potentially act as biomarkers in the monitoring of disease progression. To do so, we collected blood from a specific set of paediatric control subjects, patients with T1D at onset, at 1st year of evolution and at 2nd year of evolution, and their clinical data is summarised in **Table 26**. No differences were found in age nor BMI between the groups. Significant differences

Table 26. **Data from the paediatric patients with T1D and control subjects recruited for the study**

Parameter	Paediatric control subjects	Paediatric patients with T1D		
		At onset	At 1st year of evolution	At 2nd year of evolution
N	10	11	11	6
Gender	6/10 (60%) Female 4/10 (40%) Male	6/11 (55%) Female 5/11 (45%) Male	8/11 (73%) Female 3/11 (27%) Male	5/6 (83%) Female 1/6 (17%) Male
Age (years)	7.36 ± 2.52	8.35 ± 5.92	10.73 ± 3.73	10.02 ± 2.78
BMI (kg/m^2)	17.42 ± 2.19	17.19 ± 3.05	17.94 ± 2.59	18.13 ± 2.23
Age at T1D diagnosis (years)	NA	8.35 ± 5.92	10.17 ± 3.70	8.45 ± 2.73
Duration of T1D (years)	NA	0.00 ± 0.00	0.56 ± 0.18****	1.57 ± 0.10****/++++
HbA1c (%)	NP	10.57 ± 2.05	7.25 ± 0.99****	8.30 ± 1.17*
HbA1c (mmol/mol)	NP	92.05 ± 22.36	55.79 ± 10.78****	67.21 ± 12.76*
Fasting C-peptide (ng/mL)	NP	0.40 ± 0.40	<0.1 in 6/11 (55%) patients 0.72 ± 0.90	<0.1 in 3/6 (50%) patients 0.27 ± 0.06
Stimulated C-peptide (ng/mL)	NP	0.98 ± 1.02	NP	NP
Insulin dose (IU/kg/day)	NA	0.70 ± 0.19	0.60 ± 0.24	0.96 ± 0.18*/++

Data presented as mean ± SD; *p*-value calculated from Mann-Whitney test (*p<0.05, ****p<0.0001 when comparing paediatric patients at onset group with paediatric patients at 1st or 2nd year of evolution; ++p<0.01, ++++p<0.0001 when comparing paediatric patients at 1st year of evolution with those at 2nd year of evolution). T1D: type 1 diabetes; BMI: body mass index; HbA1c: glycated haemoglobin; NA: not applicable; NP: not performed.

were found in the duration of T1D between patients at onset and at 1st year of evolution (p<0.0001), between patients at onset and at 2nd year of evolution (p<0.0001), and between patients at 1st year and at 2nd year of evolution (p<0.0001), as expected. Moreover, patients at onset were found to have higher levels of HbA1c (both in percentage and in mmol/mol) than patients at 1st year (p<0.0001) and at 2nd year of evolution (p<0.05). Finally, patients at 2nd year of evolution required a higher insulin dose than patients at onset (p<0.05) and than patients at 1st year of evolution (p<0.01).

We analysed different subsets of DCs in peripheral blood of these paediatric subjects by flow cytometry, following the gating strategy displayed in **Supplementary Figure 10**. As shown in **Figure 32** (1st row, left panel), the percentage of total $CD45^+CD3^-CD19^-CD56^-CD14^-HLA\text{-}DR^+$ DCs exhibited a decrease in the 1st year of evolution in comparison to control subjects (p<0.05), although at 2nd year the levels were re-established (p<0.01). This decrease in percentage at 1st year of evolution was also reflected in absolute counts (right panel), when comparing cells/μL of total DCs in patients at 1st year with control subjects (p<0.05). Using the CD11c and CD123 markers, we studied myDCs, pDCs and the remaining subset of putative DCs that are $CD11c^-CD123^-$. Regarding myDCs ($CD11c^+CD123^-$, 2nd row, left panel), patients at 1st year of evolution displayed an increase in percentage when compared to patients at onset (p<0.05) and patients at 2nd year of evolution (p<0.01). Also, patients at 1st year of evolution had a greater percentage of myDCs than control subjects (p<0.01). None of these changes was reflected in absolute counts (right panel). As for pDCs ($CD11c^-CD123^+$, 3rd row, left panel), the percentage of these cells exhibited an increase in patients at 1st year of evolution when compared to patients at onset (p<0.05) and patients at 2nd year of evolution (p<0.05). Control subjects displayed a trend to have a greater percentage of pDCs than patients at onset (p=0.06). Regarding absolute counts of pDCs (right panel), control subjects had a higher number of these cells than patients at onset and patients at 1st year of evolution (p<0.05). Finally, regarding the subset of putative DCs that are $CD11c^-CD123^-$ (4th row, left panel), patients at 1st year of evolution had a decreased percentage of these cells when compared to control subjects (p<0.05), to patients at onset (p<0.05) and to patients at 2nd year of evolution (p<0.01). These changes were also present in absolute counts (right panel), and so patients at 1st year of evolution displayed lower counts of $CD11c^-CD123^-$ cells when compared to control subjects (p<0.01) and patients at 2nd year of evolution (p<0.05), and the same trend was observed when compared to patients at 1st year of evolution.

Due to the great number of changes observed in DCs subsets during the 1st year of evolution of the T1D, we investigated whether these alterations were due to a relevant event that takes place during this period: the PR. When considering those

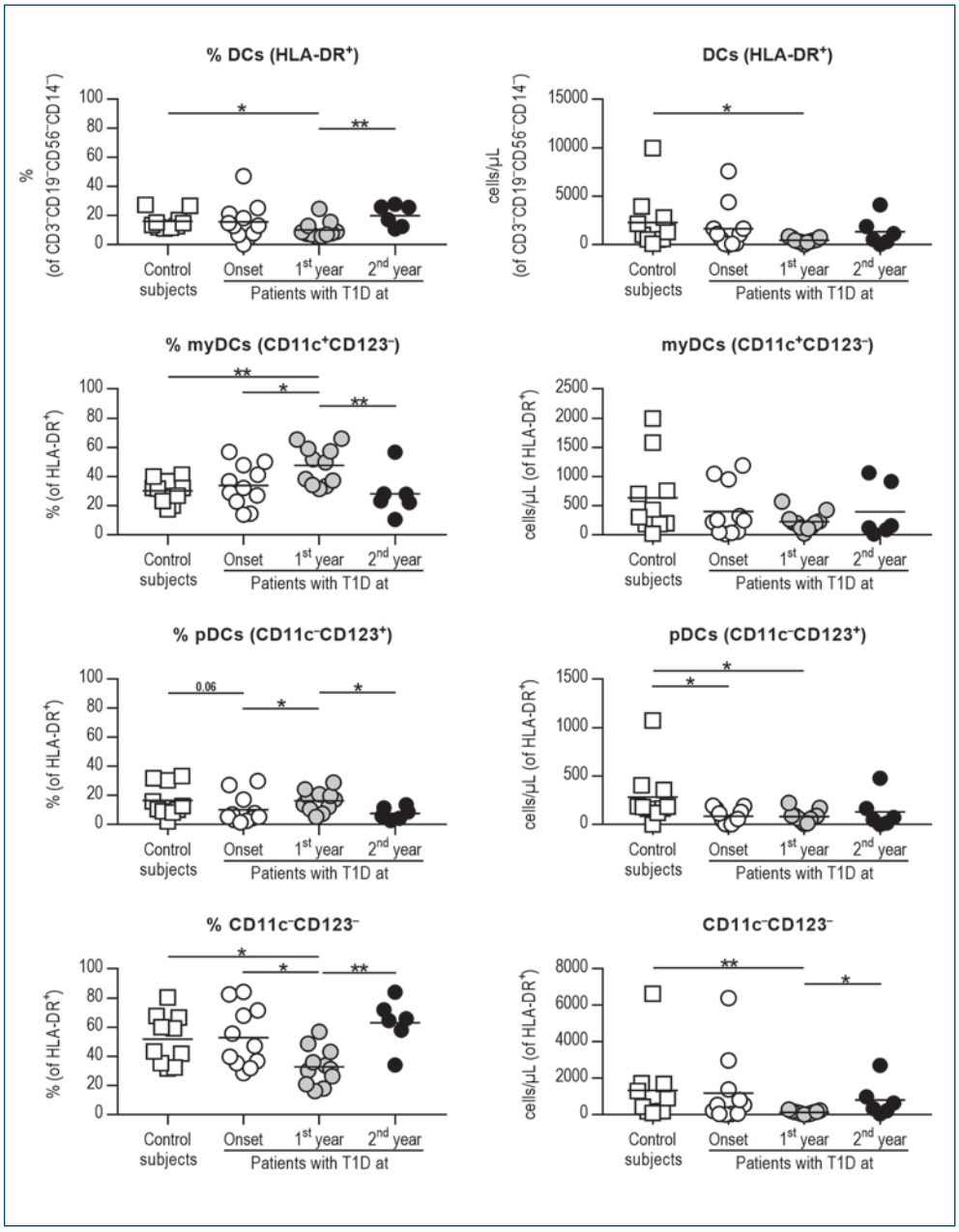


Figure 32. ***Paediatric patients with T1D show alterations in DCs subsets at different stages of the disease.*** *Percentages (left panel) and concentration (cells/µL, right panel) of total DCs (1st row, HLA-DR+ of CD3−CD19−CD56−CD14− cells), myDCs (2nd row, CD11c+CD123− of HLA-DR+ cells), pDCs (3rd row, CD11c−CD123+ of HLA-DR+ cells) and the CD11c−CD123− subset (4th row, CD11c−CD123− of HLA-DR+ cells). White squares represent control subjects (n=10), and circles represent paediatric patients with T1D at different stages of the disease: onset (n=11), white; at 1st year of evolution (n=11), grey, and at 2nd year of evolution (n=11), black. Statistically significant differences were found when comparing the groups (*p<0.05, **p<0.01, Mann-Whitney test).*

patients who were undergoing PR (meaning IDDAA1c<9), a slight trend for these patients to have lower counts of CD11c⁻CD123⁻ cells was observed (**Figure 33**, right panel) when compared to their counterparts not undergoing PR. No other differences or trends were found when comparing the percentage of these cells between patients undergoing and not undergoing PR (left panel), nor in the other DCs subsets (**Supplementary Figure 11**).

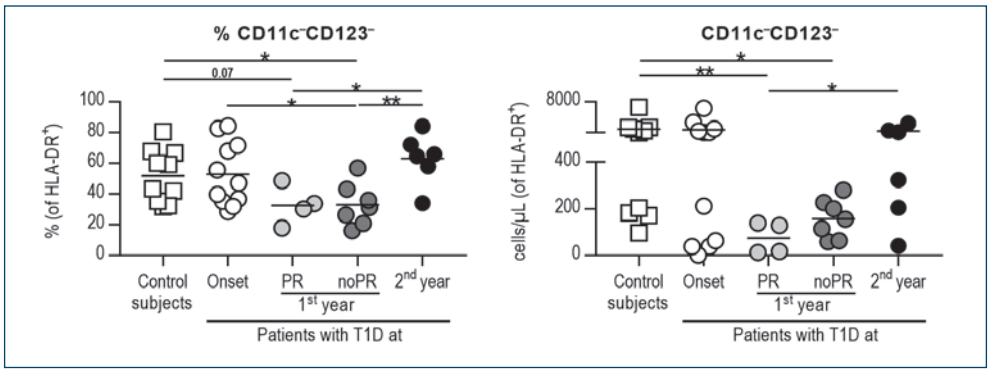
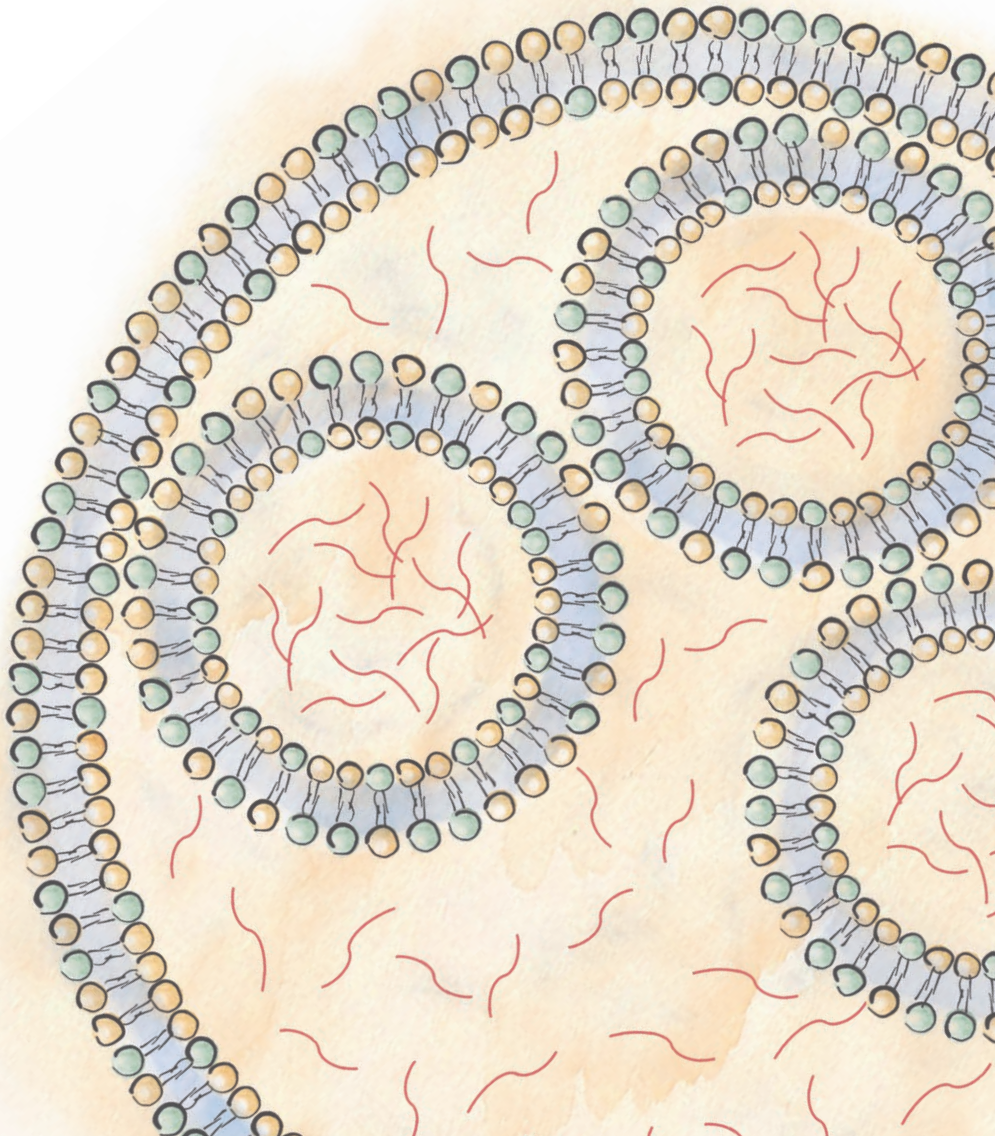


Figure 33. ***Paediatric patients with T1D at 1st year of evolution of disease undergoing PR show a slight tendency to have lower numbers of CD11c⁻CD123⁻ cells than their counterparts.*** *Percentages (left panel) and concentration (cells/µL, right panel) of the CD11c⁻CD123⁻ subset (CD11c⁻CD123⁻ cells of CD45+CD3⁻CD19⁻CD56–CD14⁻HLA-DR⁺ cells). White squares represent control subjects (n=10), and circles represent paediatric patients with T1D at different stages of the disease: onset (n=11), white; undergoing PR at 1st year of evolution (n=4, PR), light grey; not undergoing PR at 1st year of evolution (n=7, noPR), dark grey, and at 2nd year of evolution (n=11), black. Statistically significant differences were found when comparing the groups (*p<0.05, **p<0.01, Mann-Whitney test).*

DISCUSSION



Autoimmune diseases are prompted by defective immunological tolerance against the host's own cells and tissues. Currently, more than 80 autoimmune diseases have been described, collectively affecting almost 10% of the global population. Despite the involvement of multiple but similar underlying mechanisms and the many different targeted organs, autoimmune diseases are often chronic and disabling, lead to loss of organ function and require high medical expenses (Ramos *et al.*, 2015). Fortunately, in the last few years, many efforts have culminated with the implementation of therapies that have revolutionised the fate of the subjects living with them. In the best scenario, these treatments have transformed autoimmune diseases into manageable chronic ones, either by providing replacement therapy or by hampering the pathogenic actors responsible for their development and maintenance. However, these treatments are not exempt from mild and severe side effects, which can affect the normal development of an immune response against other antigens, or even other vital physiological processes. That is why immunotherapies able to re-educate the immune response and to re-establish immunological tolerance are of paramount importance. These immunotherapies should specifically involve only those autoantigens for which tolerance has been breached, avoid side effects and allow for the regeneration of the targeted tissue and mass preservation. It is also crucial that these immunotherapies only correct the misguided autoimmune response and leave untouched other immunosurveillance mechanisms, as well as induce immunological memory to ensure that autoimmunity against the same antigen does not recur. In the current scenario, many strategies aiming to restore tolerance in an antigen-specific manner have thoroughly been explored in the preclinical setting and have now entered clinical trials with a confident stride. Under the most favourable conditions, one of these approaches will mark an utmost breakthrough in the field of immunological tolerance re-establishment. Notwithstanding, even if a robust safety profile is confirmed but only a modest clinical efficacy is achieved, these approaches will undoubtedly provide further insight into the pathogenic mechanisms of autoimmune diseases, will enormously expand the knowledge in the field and will smooth the path for future studies into the definitive re-induction of immune tolerance.

In the matter of successful and permanent tolerance induction, nature has an immense head start on researchers. Aside from the delicate mechanisms governing central and peripheral tolerance, which are dedicated to hindering autoreactivity, the organism employs other physiological processes to induce tolerance to autoantigens and avoid the so-called *horror autotoxicus* —a term coined by the bacteriologist and immunologist Paul Ehrlich at the beginning of the 20th century which referred to the body's innate *horror* to immunological self-destruction. One of such is the process called efferocytosis or uptake of apoptotic cells carried out by APCs (Szondy *et al.*, 2017; Vives-Pi *et al.*, 2015). Millions of cells die via apoptosis or programmed cell death every second, but homeostasis is maintained due to the rapid and inflammatory-free disposal of the massive number of cell corpses. These apoptotic cellular remnants become a substantial source of autoantigens for DCs to present in a tolerogenic manner, so that they can be recognised as "self" and impede the development of autoimmune responses (Erwig and Henson, 2007). On the other hand, deficient efferocytosis leads to the transformation of apoptotic cells into secondary necrotic cells, and it is their own released danger signals and pro-inflammatory milieu that enable the maturation of DCs and the immunogenic presentation of autoantigens. Interestingly, this phenomenon has been proposed as one of the first events in autoimmune diseases (Abdolmaleki *et al.*, 2018; Martinez, 2017; Wickman *et al.*, 2012).

To reverse the pathological process of autoreactivity, our group opted to fight fire with fire by taking advantage of the inherent immunomodulatory features of apoptotic cells to recover tolerance to β-cells in a T1D setting. As a proof of concept, apoptotic β-cells were loaded into DCs, leading to the acquisition of tolerogenic features by these APCs (Marin-Gallen *et al.*, 2010). In the context of this antigen-specific cellular immunotherapy, the release of PGE_2 was demonstrated as partly responsible for the acquisition of immunosuppressive ability (Pujol-Autonell *et al.*, 2013). Furthermore, when this cellular therapy was administered to prediabetic NOD mice, it significantly decreased the incidence of the disease (Marin-Gallen *et al.*, 2010), thus confirming the raw potential of efferocytosis and tolDCs to re-establish antigen-specific tolerance. However, the obtaining, preservation and standardisation of human autologous apoptotic β-cells to translate this strategy into the clinics was almost inconceivable, and so surrogate apoptotic β-cells were required for its implementation. An original and cost-competitive way to mimic their particular features was to generate synthetic phospholipid-bilayered nanovesicles —liposomes— rich in PS, an *Eat me* signal that is exposed in the plasma membrane of apoptotic cells, and to load them with insulin peptides to direct the tolerance restoration process. Remarkably, this simple but ingenious strategy proved equally effective in generating tolDCs and preventing experimental T1D, but with the additional benefit that liposomes are easy to mass-produce, standardise and adapt to the requirements of each patient if necessary.

The PS-liposomes strategy relies knowingly on apoptotic mimicry. As aforementioned, the clearance of apoptotic cells is an *immunologically-quiet* homeostatic process, which is devoid of any concurrent inflammatory or immunogenic response. The externalisation of PS on the apoptotic cell membrane allows for the phagocyte to engulf said cell, and also acts as an immunosuppressive signal to promote tolerance. In fact, PS is able to induce specific intracellular signalling in phagocytes and the secretion of anti-inflammatory mediators, achieving a successful dampening of the local inflammation (Freire-de-Lima *et al.*, 2006; Huynh *et al.*, 2002). Therefore, it comes as no surprise that pathogens and cancerous processes —which an immunogenic response could very well thwart— also employ apoptotic mimicry to escape immunosurveillance mechanisms and thrive. For instance, externalised PS has been hijacked by enveloped viruses such as Vaccinia, Cytomegalovirus, Lentivirus, Dengue and Ebola, and non-enveloped viruses, such as Hepatitis A and Polio, cloak themselves in cell-derived PS-rich vesicles with the sole aim to promote virus internalisation and evade the immune response (Birge *et al.*, 2016). On the same note, viable tumour cells can externalise PS and release PS-rich exosomes, which act as immunosuppressive agents and aid in the creation of a local immunosuppressive environment to escape immune detection (Bondanza *et al.*, 2004; Kelleher *et al.*, 2015). Indeed, the fact that both pathogens and tumour cells "have learned" to use PS and apoptotic mimicry to their advantage speaks of the importance of efferocytosis as a tolerance-inducing process. Similarly, our ultimate goal with the use of PS-liposomes is none other than to make use of apoptotic mimicry to restore tolerance in autoimmune diseases. For this reason, PS-liposomes have been generated with specific physicochemical features and components that operate coordinately and seamlessly towards this objective.

Concerning our liposome strategy, all liposome preparations were generated to have a diameter greater than 500 nm to allow efficient phagocytosis and a multilamellar and multivesicular vesicle morphology, which confers advantages in terms of encapsulation and immunological activity (Watson *et al.*, 2012). Regarding their composition, PS-liposomes were prepared with two phospholipids (PS and PC) and CH, which was incorporated for stability purposes (Kersten and Crommelin, 1995). The mean particle size of PS-containing liposomes ranged between 500 nm and 1,000 nm, and the surface charge was always negative (oscillating between –30 mV and –40 mV) due to the presence of PS. Additionally, this anionic charge allowed PS-liposomes to be less immunogenic than neutral or positively-charged liposomes (Nakanishi *et al.*, 1999). By contrast, PS-free PC-liposomes were only prepared with PC and CH, ranged between 900 nm and 2,000 nm in diameter and exhibited a more neutral surface charge (>–10 mV). Precisely the lack of PS and large diameter made PC-liposomes very unstable, and their sedimentation was observed after

only 1 hour of preparation. Concerning the peptide encapsulation efficiency, the amino acid composition, charge and solubility of each peptide could have determined their yield of encapsulation into PS-liposomes. For example, MOG_{40-55} peptide and human insulin B chain were encapsulated with an optimal 90% yield, whereas 2.5mi peptide, NRP-V7 peptide and human insulin A chain were only loaded into the liposomes with a 20-50% yield. On another note, regardless of the loaded peptide, the peptide encapsulation efficiency of PS-liposomes was always heightened in comparison to that of PC-liposomes. One explanation could be that the negative surface charge of PS-liposomes favourably attracted neutral or positively-charged peptides, in contrast to the neutral surface of PC-liposomes. These facts, together with the thoroughly proven anti-inflammatory effect of PS (Chen *et al.*, 2004; Glassman *et al.*, 2018; Harel-Adar *et al.*, 2011; Ramos *et al.*, 2007; Shi *et al.*, 2007), raises PS as a very powerful companion and agent to deliver autoantigenic peptides to DCs, by conferring stability to those liposomes as well as providing them with a dominant tolerogenic signal. Thus, PS-liposomes do not only act as vehicles to deliver the encapsulated peptide, but also target DCs and phagocytes with functional PS receptors, which are able to phagocyte particles larger than 500 nm. Also, the efficient encapsulation of several peptides and preservation of lipid concentration, diameter and charge turn the PS-liposomes strategy into one largely adaptable and versatile, and confers the potential for this strategy to be used as a platform in the tackling of several antigen-specific autoimmune diseases. Moreover, these liposomes protect the encapsulated peptides from external degradation and, more importantly, from immunogenic processing and recognition by several cells and molecules of the immune response.

Logically, autoantigen-loaded PS-liposomes are primarily targeted to APCs with the objective that they relay their tolerogenic message to T lymphocytes, and that they do so in an antigen-specific manner. Thus, to determine the nature of this antigen-specific $CD4^+$ T cell expansion emerged as one vital aspect for our immunotherapy, and the MHC class II tetramer analysis allowed for the direct identification of antigen-specific $CD4^+$ T cells and the assessment of their phenotype. Since we already knew that the antigen-specific $CD4^+$ T cell expansion was observed in the $FoxP3^+$ and $FoxP3^-$ compartments (Pujol-Autonell *et al.*, 2015), we interrogated whether the liposomal strategy could also expand non-classical TR1 cells. After confirming that, in our setting, the treatment with PS2.5mi-liposomes and PC2-5mi-liposomes also resulted in a fast expansion of antigen-specific $CD4^+$ T cells, which is typical for a memory response (Panus *et al.*, 2000), we assessed the level of expression of CD49b and LAG3 markers in these $2.5mi^+CD4^+$ T cells, and compared them to those of non-antigen specific $2.5mi^-CD4^+$ T cells. Regardless of the liposome treatment used, the marker CD49b was expressed at higher levels in $2.5mi^+CD4^+$ T cells than

in 2.5mi^{-}CD4^{+} T cells, and the same trend was observed for the LAG3 marker. Interestingly, this expansion of putative TR1 cells is achieved with only a 50% of 2.5mi peptide encapsulation efficiency into PS-liposomes. Indeed, these results suggest that the treatment could be inducing TR1 cells as well as classical FoxP3^{+} Treg cells. One feasible way to confirm this hypothesis would be to determine whether the putative 2.5mi^{+}CD4^{+} TR1 cells are negative for FoxP3 expression by employing NOD.FoxP3^{EGFP} reporter mice, which express the green fluorescent protein (GFP) under the control of the *Foxp3* gene promoter, thus allowing for the selection of FoxP3^{-} cells for further analysis. Furthermore, we could asses the ability of 2.5mi^{+}CD4^{+} T cells to secrete IL-10, since it is widely accepted that TR1 cells secrete that cytokine to enhance immunoregulation (Gagliani *et al.*, 2013). Nevertheless, it is reasonable to speculate that the expanded CD4^{+} T cells do have a regulatory function, as otherwise the disease would have been accelerated when autoantigen-loaded PS-liposomes were administered to prediabetic NOD mice in the preventive assay (Pujol-Autonell *et al.*, 2015). Similarly, other authors have reported the generation of TR1 cells after antigen-specific nanotherapeutic treatment in NOD mice, and these cells are accountable for the beneficial effect of the immunotherapy (Clemente-Casares *et al.*, 2016). And yet, we cannot rule out that other subsets of antigen-specific FoxP3^{-} Treg cells might be contributing to restoring tolerance to islet antigens, for which specific markers might not be even described yet.

What was unexpected, however, was that PC2.5mi-liposomes could also prompt a high expression of CD49b and LAG3 markers in 2.5mi^{+}CD4^{+} T cells. In our early reasoning, albeit simplistic, it seemed logical to think that PS2.5mi-liposomes would induce the expansion of Treg cells whereas PC2.5mi-liposomes would elicit the proliferation of non-regulatory T cells. Seeing as PC2.5mi-liposomes induced similar expansions of FoxP3^{+} and FoxP3^{-} T cells (Pujol-Autonell *et al.*, 2015) and putative TR1 cells in comparison to PS2.5mi-liposomes, other markers should be employed to test their hypothetical regulatory action, such as the secretion of IL-10. Additionally, the testing of the preventive potential of autoantigen-loaded PC-liposomes *in vivo*, in NOD mice, would constitute the ultimate proof for the assertion of PS as the actual immunomodulatory agent in our setting.

Because CD8^{+} T cells are responsible for the destruction of β-cells, we also determined the effect of the liposomal therapy on antigen-specific CD8^{+} T cell frequency. Using MHC class I tetramer analysis, we confirmed that PSNRP-liposomes treatment also induced a fast expansion of NRP^{+}CD8^{+} T cells in the three secondary lymphoid organs analysed. In this case, the administration of PCNRP-liposomes induced the expansion of NRP^{+}CD8^{+} T cells as well, but at a lesser extent than PSNRP-liposomes, especially in PLN and MDLN, which points to the differential effect of PS in driving antigen-specific CD8^{+} T cell expansion. Also, apart from the sham control,

we included an NRP-V7 peptide control in these experiments, showing that the encapsulation of the peptide in PS-liposomes is much more potent in inducing a clonal expansion than the peptide alone, even if the efficiency of this encapsulation is only 20%. At this point, it is daring to assume that these antigen-specific $CD8^+$ T cells could have a regulatory function but, following the previous reasoning proposed for $CD4^+$ T cells, $CD8^+$ T cells expanded by the treatment cannot have a pathogenic role either. In recent studies, $CD8^+$ Treg cells have been described to express the *FoxP3* gene, express LAG3 and CTLA-4 molecules on their membrane and secrete IL-10 and TGF-β, among other features (Vieyra-Lobato *et al.*, 2018), and so studying these would help us gain insight into this subset. In this sense, $FoxP3^+CD8^+$ Treg cells reportedly delay skin graft rejection and graft versus host disease in humanised mice (Bézie *et al.*, 2018) and mitigate the disease severity in humans (Beres *et al.*, 2012). Similarly, in patients with lupus nephritis, $FoxP3^+CD8^+$ Treg cells have been induced after immunomodulatory treatment and exerted a beneficial effect in disease activity (Tsai *et al.*, 2014). Even if the antigen-specific $CD8^+$ T cells expanded by our liposomal treatment were revealed as $FoxP3^–$, other authors have demonstrated the protective role of the $FoxP3^–CD8^+$ subset in the setting of experimental T1D after nanoparticle treatment (Tsai *et al.*, 2010). Therefore, assessing the nature of the expanded antigen-specific $CD8^+$ T cells in terms of FoxP3 expression as well as other regulatory mechanisms would prove invaluable to elucidate the biological and cellular effects prompted by the herein proposed immunotherapy.

However, we cannot disregard that the immunotherapy only achieves a maximum of a 2-3% expansion of antigen-specific T cells, either $CD4^+$ or $CD8^+$, and that we may not be capable of identifying these refined mechanisms with such low numbers. Then, one feasible option would be to turn to specific strains of transgenic mice generated into the NOD background which carry particular TCR transgenes, leading to most of the T cells expressing these distinct TCRs. For instance, the BDC-2.5mi mice harbour the rearranged *Tcra* and *Tcrb* transgenes from the diabetogenic BDC-2.5mi $CD4^+$ T cell clone (Katz *et al.*, 1993), whereas the 8.3-NOD mice carry the rearranged *Tcra* and *Tcrb* transgenes from the NY8.3 $CD8^+$ T cell clone (Verdaguer *et al.*, 1997). Indeed, many efforts need to be dedicated to this particular aspect of the immunotherapy before it can be translated to humans, since it is vital that we find the exact mechanism by which it exerts its biological function. Furthermore, it would be ideal if we could identify a cellular biomarker of its efficacy which would allow us to monitor the switching of the autoimmune response against β-cells towards a tolerogenic profile.

Two other vital issues for the successful implementation of the PS-liposomes immunotherapy in the clinics are its potential toxicity and tolerability. To address this, we first tested 14 daily injections of PS-liposomes at 30 mM of lipid concentration on

prediabetic NOD mice, which revealed a well-tolerated and safe profile. Only mild or minor complications were found, which were distributed likewise among the sham and treated groups; therefore, it is more likely that they were due to the significant amount of injections the mice received during this study. Also, the loss of 10% of body weight reported in 1 mouse of the sham group and 2 of the treated group could indicate that these mice were on the road to developing diabetes promptly, although none of the mice tested positive for glucosuria. The biochemical analysis of the serum did not reveal any significant alterations due to the PS-liposomes treatment either, nor in lipid or cholesterol profiles nor in liver enzymes or metabolites. Additionally, one single dose of PS-liposomes at 90 mM of lipid concentration was well-tolerated and safe as well, thus indicating that PS-liposomes are not toxic nor harmful *in vivo*.

Still, as encouraging as these results are, we are fully aware that they are preliminary and that more animals should be included in future studies to validate the tendencies observed. At the moment, it is indeed challenging to extrapolate these results to human. In the current landscape, many formulations of liposomes have been approved for a wide range of purposes (Bulbake *et al.*, 2017), and they are mainly administered through the i.v. and i.m. routes (Zylberberg and Matosevic, 2016), in a wide range of dosages and displaying a rather small size (<100-300 nm) (Bulbake *et al.*, 2017). Taken together, these data point to the overall safety of liposomal therapy. Furthermore, in specific cases, they have even reduced the toxicity of the drug they encapsulate by facilitating its accumulation only in the target tissue, as is the case of the pegylated liposomal doxorubicin or liposomal cisplatin (Sousa *et al.*, 2018). It is worth mentioning that, to date, the only commercially available liposomal formulation containing PS is the osteosarcoma-indicated drug Mepact® (Takeda Pharmaceutical Company Ltd). This drug is administered i.v., and is composed of <100 nm multilamellar liposomal vesicles encapsulating mifamurtide —a synthetic derivative of the muramyl dipeptide of the *Mycobacterium* family (Bulbake *et al.*, 2017). These liposomes are made of PC and PS to target phagocytes, and so PS does not function as a tolerogenic agent by any means. Therefore, many studies will have to be performed before our PS-liposomes immunotherapy can make the final translational leap, in which the route of administration, posology and dosage will have to be carefully studied and optimised, using, perhaps, bigger animals than mice.

After confirming that PS-liposomes loaded with β-cell autoantigens could arrest the autoimmune aggression to β-cells in an antigen-specific manner, our next logical question regarded the potential of this PS-liposomes immunotherapy to act as a platform to re-establish tolerance in several antigen-specific autoimmune diseases by only replacing the encapsulated autoantigenic peptide. To do so, we turned to MS, an autoimmune disease in which mainly myelin autoantigens are attacked. We considered this disease an excellent place to start, since the autoantigens fuelling

the autoimmune response are largely known and its experimental model —the EAE— is widely established and used (Bjelobaba *et al.*, 2018). In this context, we demonstrated that DCs from EAE-induced animals captured PS-liposomes in a rapid and active manner *in vitro*. Since the engulfment of PS-liposomes was only performed at 37 °C and not at 4 °C, it is reasonable to theorise that the DCs need to be metabolically active to perform the capture of PS-liposomes, and the kinetics of this process concurs with the active mechanism of phagocytosis mediated by PS receptors (Segawa and Nagata, 2015). One other data to support the entry of PS-liposomes into DCs by phagocytosis is their large diameter (>500 nm), as compared to the smaller size (<100 nm) of fluids and solutes of the extracellular bulk that enter the cell through pinocytosis (Lim and Gleeson, 2011).

Afterwards, PS-liposomes were loaded with the immunodominant peptide of the MOG protein (MOG_{40-55}), which had priorly been used to induce the disease in the mice. Firstly, DCs cocultured with PSMOG-liposomes displayed optimal levels of viability, confirming once more the safe profile of the PS-liposomes therapy. Secondly, after PSMOG-liposomes engulfment, DCs preserved the expression of MHC class II molecules when compared to iDCs, decreased CD86 expression and increased CD40 expression, albeit their levels were far lower than those of mDCs. These data pointed to the acquisition of a semimature phenotype by DCs after the phagocytosis of PSMOG-liposomes, which is consistent with their putative tolerogenic capacity (Lutz and Schuler, 2002). Furthermore, the uptake of PSMOG-liposomes by DCs induced a marked secretion of PGE_2, which is in agreement with our previous studies using NOD mice (Pujol-Autonell *et al.*, 2013, 2015). Indeed, PGE_2 is reported to modulate cellular immunity (Goodwin and Ceuppens, 1983), induce Treg cell function (Baratelli *et al.*, 2005) and inhibit T cell proliferation (Ruggeri *et al.*, 2000). Moreover, PGE_2 production is elicited after apoptotic cell removal by macrophages and DCs, and is key for active suppression of inflammation (Fadok *et al.*, 1998; Pujol-Autonell *et al.*, 2013). Interestingly, DCs cocultured with empty PS-liposomes displayed a trend for producing lower amounts of PGE_2 than PSMOG-DCs, and DCs exposed to the MOG_{40-55} peptide alone produced PGE_2 in levels similar to iDCs. Thus, the production of high levels of PGE_2 by DCs after autoantigen-loaded PS-liposomes phagocytosis indicates that we could be successfully achieving apoptotic mimicry with this liposomal strategy.

Once the tolerogenic potential of PS-liposomes in DCs was confirmed *in vitro* in the experimental MS setting, we assessed the effect of the direct PSMOG-liposomes administration into EAE-induced mice. Of note, in order to determine the effect of a treatment in EAE, three different approaches can be used: preventive, in which the therapy is administered before the MOG peptide immunisation; preclinical, in which the therapy is tested after the immunisation, and therapeutic, in which it is adminis-

tered after the onset of the disease (Mansilla *et al.*, 2015). We decided on a preclinical approach based on two premises: in the preclinical model, the autoimmune reaction has already been unfolded —in a setting analogous to that of NOD mice—, and so PS-liposomes would induce *tolerance re-establishment* rather than *tolerance per se*. Importantly, PSMOG-liposomes treatment was successful at arresting the autoimmune aggression to myelin antigens *in vivo*, thereby reducing the incidence of the disease and its severity —cumulative score and maximum score— when compared to the sham and empty PS-liposomes control groups. Additionally, there was a trend for a slight delay in the onset day and an improvement in the survival rate of the mice treated with PSMOG-liposomes, and they did not endure a decrease in their body weight as markedly as the mice in the control groups did. On another note, the treatment with MOG peptide alone resulted in an apparent amelioration of the disease, a finding that has been reported in previous studies (Critchfield *et al.*, 1994; Devaux *et al.*, 1997; Jiang *et al.*, 2009; Wang *et al.*, 2016). Nonetheless, the effect of MOG peptide treatment appeared to be minor than the induced by PSMOG-liposomes, which reflects the synergistic action of both PS and MOG peptide in tolerance re-establishment. In a similar manner, the combination of MOG peptide with other compounds, such as vitamin D or rapamycin, shows synergism in ameliorating EAE (Chiuso-Minicucci *et al.*, 2015; Tostanoski *et al.*, 2016). Also, as expected, empty PS-liposomes did not provide any manner of protection in front of EAE disease, hence demonstrating that the antigen-specificity is an integral and indispensable feature for the successful restoration of immunological tolerance using this approach. These results are consistent with previous data obtained in the studies employing NOD mice, in which tolDCs loaded with apoptotic cells from an irrelevant cell line and empty PS-liposomes were unable to arrest β-cell autoimmunity (Pujol-Autonell *et al.*, 2013, 2015).

Since the timely administration of PSMOG-liposomes before the appearance of the clinical signs achieved a significant reduction in the incidence and the severity of EAE, we hypothesised that this long-term specific tolerance re-establishment was probably grounded on the generation of regulatory mechanisms elicited by DCs after PS-liposomes treatment. That is why we determined the frequency and absolute counts of $CD4^+CD25^+FoxP3^+$ T cells on the animals treated with PSMOG-liposomes. Contrary to our initial expectations, the frequency of $CD4^+CD25^+FoxP3^+$ T cells was decreased in favour of an increase in $CD4^+CD25^+FoxP3^-$ T cells in comparison to sham mice. Additionally, the frequency of the $FoxP3^-$ subset correlated with a milder clinical score in EAE-induced mice treated with PSMOG-liposomes, but no correlation was found between the frequency of the same subset and the clinical score in sham mice. Therefore, it is not far-fetched to speculate that these cells, which are actively generated after the treatment with autoantigen-loaded PS-liposomes, could

have a regulatory function, or that they contribute to tolerance re-establishment somehow. Following the reasoning exposed for the study of antigen-specific $CD4^+$ T cells in experimental T1D, we wondered whether this treatment could be inducing a TR1 cell expansion in this model as well. Thus, we studied the expression of CD49b and LAG3 markers on T cells, determining that the LAG3 marker was significantly increased in $CD4^+$ T cells from mice treated with PSMOG-liposomes and that the CD49b displayed the same trend, pointing to the generation of TR1 cells after PS-liposomes treatment. However, we cannot ignore that these results are not sufficient to affirm that an expansion of TR1 cells took place after PS-liposomes administration. To do so, we would have to assess MOG-specific T cells, their levels of FoxP3, CD49b and LAG3 expression and their functionality in terms of IL-10 production. Nonetheless, the expression of LAG3 has been described as sufficient to confer regulatory activity to $CD4^+$ T cells (Huang *et al.*, 2004), its engagement with MHC class II inhibits DC immunogenic activation (Liang *et al.*, 2008), and its absence precipitates the development of T1D in NOD mice with 100% of penetrance (Bettini *et al.*, 2011). Similarly, in EAE disease, other authors have made evident the regulatory function of TR1 cells (Barrat *et al.*, 2002; Wildbaum *et al.*, 2002), and other nanotherapeutic agents have been reported to induce TR1 cells in the same setting (Clemente-Casares *et al.*, 2016). Finally, the fact that suggestive TR1 cells are expanded after PS-liposomes treatment in both experimental T1D and MS models does nothing but uphold the possibility that TR1 cells are indeed the subset responsible for the arrest of autoimmunity after PS-liposomes administration.

It is indeed very reassuring that our PS-liposomes approach has yielded encouraging results in two animal models of autoimmune diseases. Although further research is essential to determine the mechanism by which PS-liposomes restore immunological tolerance, the fact remains that they are able to successfully and permanently arrest autoantigen-driven reactivity in, at least, two preclinical settings. This pushes us to believe that this biomimicry strategy, which is solely based on the physiological tolerance-inducing process termed efferocytosis, could indeed be used as a platform in many situations where tolerance re-establishment is needed by co-delivering a binary signal of tolerance and specificity. However, our ultimate goal is not to tackle autoimmune diseases in mice but in humans. Preclinical studies using experimental rodent models are of invaluable importance and necessary to ensure the safety and the efficacy of the tested strategy prior to clinical use, but they present significant limitations. To begin with, there are significant differences in the size, the anatomical distribution and the immune system of mice and humans (Mestas and Hughes, 2014). Also, experimental models are usually established in syngeneic strains, which is antagonistic to the high diversity inherent to the human species. Furthermore, these models are used for the similarities between the dis-

ease they suffer and the human disease, but they seldom reproduce exactly the same disease: e.g., the disease in the model might need to be induced instead of being spontaneous, and its course and severity may be far apart from those of the human disease. These differences must be recognised and considered in order to translate promising strategies to the clinics, and so, primarily, experimental models serve to establish a *proof of concept*.

That is why, aiming for the clinical potential of our PS-liposomes strategy, we encapsulated human insulin peptides in order to assess the effect of autoantigen-loaded PS-liposomes in human DCs from patients with T1D *in vitro*. First, we reached for the Endocrinology section of the Germans Trias i Pujol Hospital to recruit adult patients who had a minimum of 6 months of disease progression, in order to avoid the dysglycaemia that the onset phase and exogenous insulin introduction entail. We then recruited age-matched and gender-matched control subjects, and so no differences in the personal or clinical data were found between the groups. Monocytes were isolated from PBMCs and differentiated into DCs with optimal yield, purity and viability. Most importantly, the monocyte isolation and DC differentiation behaved similarly in patients and control subjects. Thus, the effect of PS-liposomes in DCs was determined in several aspects: toxicity, phagocytosis, phenotype, cytokine profile, T cell proliferation induction, and transcriptomics.

Firstly, PS-liposomes did not affect the viability of human DCs by any means in any of the conditions studied, validating the robust safe profile they had already proven in the animal models. Following this, we explored the nature and kinetics of engulfment in which human DCs captured PS-liposomes. DCs from patients and control subjects uptook PS-liposomes only at 37 °C —and not at lower temperatures—, meaning that human DCs captured PS-liposomes through active phagocytosis, which is consistent with the previous results obtained in the NOD and EAE models (Pujol-Autonell *et al.*, 2015, 2017). Additionally, we interrogated the role of PS in the driving of liposome phagocytosis by employing PS-free liposomes (PC-liposomes). We are well aware that this constitutes an indirect approach, and that the more sensible one would have been to block PS receptors on DC membranes, but it was impractical to do so knowing that phagocytes possess an almost endless number of PS receptors that can probably act simultaneously, cooperatively and pleiotropically (Moller-Tank and Maury, 2014). In this way, we demonstrated that lipid membrane composition was crucial for the rapid engulfment of PS-liposomes by DCs, since the presence PS substantially accelerated PS-liposomes phagocytosis both in control subjects and patients with T1D. Also, PS-liposomes were more efficiently engulfed than the equivalent ones without PS in the first 2 hours of coculture, which is reminiscent of the quick and efficient apoptotic cell clearance that DCs physiologically perform. This pushes us to believe that, when encountering PS-lipo-

somes, DCs are deceived into sensing that they are actual apoptotic cells that need to be rapidly efferocyted in order to avoid secondary necrosis that could contribute to autoimmunity (Abdolmaleki *et al.*, 2018; Martinez, 2017). Nevertheless, to be sure that PS presence was the factor determining their rapid engulfment, we studied the kinetics of phagocytosis of different sized PS-liposomes and PC-liposomes, which were indeed similar regardless of the size, thus confirming that PS is the critical factor in accelerating PS-liposomes phagocytosis.

The second aspect was the assessment of the DCs' phenotype and cytokine profile after being cocultured with PSAB-liposomes. Of note, we encapsulated each chain of the human insulin molecule separately into two different liposomes (PSA-liposomes and PSB-liposomes) to avoid any possible biological effects of insulin, but tested them simultaneously to assess their effect as autoantigens since both peptides contain well-known β-cell specific target epitopes in human T1D (Mauvais *et al.*, 2016; Zhang *et al.*, 2008). After liposome engulfment, PSAB-DCs maintained high levels of PS receptors to mediate this uptake, suggesting that the phagocytosis ability is preserved in tolDCs. Interestingly, higher levels of TIM-4 expression were observed on DCs from patients than from control subjects, perhaps contributing to a positive feedback of phagocytosis. By contrast, the levels of PS receptors were decreased in mDCs because, after maturation, they no longer need PS receptors nor the phagocytic activity to browse or sample lipid particles (Albert *et al.*, 1998). Also, the pattern of expression of molecules involved in antigen presentation, activation and costimulation in PSAB-DCs was consistent with a tolerogenic function, being more similar to iDCs than to mDCs. As expected, this phenotype was reminiscent of the one previously observed in mice (Pujol-Autonell *et al.*, 2015, 2017). Additionally, the activation marker CD25 —which has been linked to the DCs' activation and autoimmunity (Driesen *et al.*, 2008; Wuest *et al.*, 2011)— supported the notion that PSAB-DCs displayed an intermediate activation status after PS-liposomes phagocytosis. The expression of chemokine receptors supported the ability of PSAB-DCs to migrate to secondary lymphoid tissues (Förster *et al.*, 2008; Ricart *et al.*, 2011) and, furthermore, the high CCR7 expression is associated with the induction of tolerance after efferocytosis (Steinman *et al.*, 2000). Finally, the expression of pattern recognition receptors was not altered by liposomes, as described for human DCs (Chen *et al.*, 2004). It is worth mentioning that the RNA-seq data support this regulatory phenotypic profile, since no increase in the gene expression of any the abovementioned molecules was observed after PS-liposomes engulfment.

Furthermore, upon PS-liposomes capture, DCs showed a trend for TGF-β1 secretion, this being a reported effect driven by PS (Fond and Ravichandran, 2016) with ability to suppress DC maturation and define the T cell response afterwards. By contrast, IL-10 secretion was hardly detected. As expected, PS-liposomes capture did

not induce pro-inflammatory cytokine secretion by DCs, but exposure to maturation stimuli did. We also observed trends for minor differences between DCs from patients and control subjects, which could be due to epigenetic changes caused by the existent autoimmunity and metabolic dysregulation, as aforementioned. Nevertheless, the overall profile ascertained with these results pointed to the tangible tolerogenic effect of PS-liposomes in human DCs.

Afterwards, we analysed the rate of proliferation of autologous T cell induced by PSAB-DCs, which was similar or even lower than the induced by iDCs, both in patients and control subjects and in agreement with their tolerogenic phenotype. By contrast, mDCs induced higher rates of T cell proliferation. Interestingly, PSAB-DCs from patients with T1D displayed a more significant impaired ability to induce proliferation of the $CD8^+$ T cell subset in comparison to iDCs. This result could point to a willing reduction in the T cell cytotoxic activity —which has been described as the most important effector response in human T1D (Coppieters *et al.*, 2012b; Planas *et al.*, 2010)— executed by tolDCs. Of note, we chose to assess the impairment of T cell proliferation induction capacity in autologous cocultures since this setting is more similar to the physiological one. However, we are fully aware that the mechanisms governing the induction of T cell proliferation by DCs are far too complex to elucidate in autologous cocultures, given that the rates of proliferation induced are very modest. Further studies using tetramers would be relevant to determine the antigen-specificity and the nature of the proliferating T cells in the cocultures, thus granting a better understanding of the mechanism involved. Also, the cytokines produced during the autologous T cell proliferation assays with PSAB-DCs discarded a Th1 and Th17 profile, which could be detrimental in the induction of tolerance. In fact, IFN-γ, which is involved in a Th1 response, and IL-6 secretion, which partially contributes to induce a Th17 response in T1D (Bradshaw *et al.*, 2009), remain poorly secreted in cocultures of PBMCS with PSAB-DCs. Interestingly, higher amounts of IL-6 and IFN-γ tend to be produced in mDCs cocultures from patients with T1D when compared to those of control subjects, a finding that could reflect the ongoing autoimmune reaction present in peripheral blood from patients (Seyfert-Margolis *et al.*, 2006).

However, we cannot ignore that the results obtained indicate that PSAB-DCs, when exposed to a maturation stimulus (mPSAB-DCs), might not be as stable in their tolerogenic phenotype and functionality as the murine tolDCs were (Pujol-Autonell *et al.*, 2015). For instance, mPSAB-DCs induce the highest levels of $CD4^+$ T cell proliferation, especially in patients T1D. On the one hand, we could argue that mice are syngeneic, whereas human samples have an unavoidable inherent variability that could explain the different stability profiles observed in the two species. On the other hand, the phenotype of costimulatory molecules in human mPSAB-DCs points to intermediate levels of CD40 and CD86 expression in mPSAB-DCs in comparison

to iDCs and mDCs, which is reminiscent of the semimature phenotype that tolDCs employ to induce incomplete activation of T cells (Domogalla *et al.*, 2017). In fact, other authors have used pro-inflammatory stimuli to boost the tolerogenic properties of DCs, and argue that they remain stable against maturation (Navarro-Barriuso *et al.*, 2018a; Zubizarreta *et al.*, 2019). Nevertheless, in subsequent studies, we could consider coupling the immunotherapy with other agents known for their immunosuppressive ability, such as vitamin D3, which would further skew the functionality of DCs towards a tolerogenic one and improve the potential of the immunotherapy to arrest autoimmunity.

In order to possess a global picture of changes induced by PS-liposomes phagocytosis, a transcriptomic analysis was performed in DCs from 8 patients with a short T1D duration. Of note, we decided on culturing DCs with PSAB-liposomes for 4 hours because the phagocytosis kinetics assays revealed that 80% of PS-liposomes had been captured at this time point. The RNA-seq analysis revealed a set of DEGs in PSAB-DCs, with 87% being downregulated and only 13% being upregulated. Interestingly, these changes in gene expression were apparently aimed at avoiding the maturation of DCs, and contributed to the tolerogenic antigen presentation and functionality of the DCs. One of the most hyperexpressed genes after PS-liposomes phagocytosis was the *VEGFA* gene, involved in cytokine signalling after apoptotic cell clearance (Golpon *et al.*, 2004). This molecule recruits iDCs and inhibits their functional maturation (Ellis and Hicklin, 2008). Furthermore, the *VEGF* gene increases the expression of the *TNFSF14* gene on APCs (Petreaca *et al.*, 2008), concurring with its upregulation in DCs after the capture of PS-liposomes. Also, *TNFSF14* is expressed on iDCs but not mDCs (Tamada *et al.*, 2000), upregulates the production of TGF-β by APCs (Doherty *et al.*, 2011) and controls T cell activation and proliferation (Ware and Šedý, 2011), inducing local immunosuppression (Qin *et al.*, 2013) after interacting with its ligand on T cells. Supporting this fact, apoptotic cell clearance has been described to inhibit inflammation via TGF-β and VEGF production (Yun *et al.*, 2008). Additionally, VEGFA enhances the expression of IDO (Marti *et al.*, 2014), an immunomodulatory enzyme expressed in tolDCs (Nguyen *et al.*, 2010). Fitting well with the activation of the VEGF signalling pathway, the *HHEX* gene —a repressor of the VEGF molecule and the expression of VEGF receptors (Noy *et al.*, 2010)— was downregulated after PS-liposomes phagocytosis, whereas a reported inductor of VEGF expression, the *ATF4* gene (Terashima *et al.*, 2013), was upregulated. Moreover, the hyperexpressed *SHB* gene codifies for a protein that regulates VEGF-dependent cellular migration (Holmqvist *et al.*, 2004), DC-mediated Th2 polarisation and the induction of Treg cells (Ahmed *et al.*, 2017). Furthermore, other relevant genes in the induction of immunological tolerance, such as *TNFAIP3* and *LAIR1*, were upregulated in DCs after PS-liposomes phagocytosis. The overexpres-

sion of these two genes inhibits DC maturation, and their deficiency causes the development of autoimmune and autoinflammatory diseases (Kanakoudi-Tsakalidou *et al.*, 2014; Richard-Miceli and Criswell, 2012; Vereecke *et al.*, 2009; Zhou *et al.*, 2016). In the same way, the clear hyperexpression of the *NFKBIA* gene —a subunit of the inhibitor of NF-κB (IκB)— could contribute to reducing DC maturation and inhibiting T cell activation (Herrington *et al.*, 2016; Raker *et al.*, 2015).

Regarding the cytokine signature, the transcriptomics profile agreed with the immunoregulatory action of PS-liposomes found in the phenotypic and functional experiments. First, although efferocytosis is known to induce TGF-β secretion to promote the resolution of inflammation (Fond and Ravichandran, 2016), we only found a trend for PSAB-DCs to upregulate the *TGFB1* gene, probably due to the short time span of the experiment (Dumitriu *et al.*, 2009). In fact, TGF-β1 was found in culture supernatants 24 hours after PS-liposomes phagocytosis, as aforementioned. A *sine qua non* condition for DC tolerogenic activity is the low or null production of IL-12, and indeed, nor the *IL12A* or the *IFNG* genes were found differentially expressed to iDCs at this time point, correlating well with a tolerogenic transcriptome. Also, the immunoregulatory *IFNLR1* gene was one of the few overexpressed in DCs after PS-liposomes uptake. This receptor for IFN-λ family members is upregulated in iDCs and has been reported to induce tolDCs that promote Treg cell expansion (Mennechet and Uzé, 2006). Unexpectedly, the TNF-encoding gene was upregulated in DCs after PS-liposomes phagocytosis. However, the upregulation of the *TNF* gene in our RNA-seq agrees with the upregulation of *TNFSF14* and *TNFAIP3* genes. In this sense, a critical role for TNF has been reported in human tolDCs in the induction of antigen-specific Treg cells (Kleijwegt *et al.*, 2010), its binding to the TNFR2 induces apoptosis on autoreactive T cells and activation of Treg cells (Faustman, 2018), and our previous results showed that murine tolDCs also upregulated the *TNF* gene after efferocytosis (Pujol-Autonell *et al.*, 2013). Overall, these results are consistent with the pleiotropic effects of TNF.

Furthermore, in our previous research, PGE_2 was found to be crucial in the tolerance induced by tolDCs loaded with apoptotic β-cells and with autoantigen-loaded PS-liposomes (Pujol-Autonell *et al.*, 2013, 2015, 2017). Strikingly, this pathway did not seem to be upregulated in human DCs, which could be explained by differences in the experimental design or the described divergences between mice and men. Nonetheless, our data indirectly point to the involvement of the PGE_2 pathway in human DCs after the capture of PS-liposomes. First, we found that the *HPGD* gene, involved in PGE_2 degradation (Tai *et al.*, 2002), was downregulated after PS-liposomes uptake. Second, a trend for the *PPARG* gene, which is induced by prostaglandins and is a negative regulator of pro-inflammatory cytokine expression (Nencioni *et al.*, 2002), to be upregulated was also found, although it did not reach

significance. Furthermore, PGE_2 has been described to stimulate the synthesis of VEGF (Ben-Av *et al.*, 1995), one of the most overexpressed genes in human DCs after PS-liposomes engulfment, as abovementioned. These results suggest the involvement of the PGE_2 pathway in human immunological tolerance induction by PS-liposomes engulfment. Perhaps not so peculiarly, most of the transcriptome alterations found in DCs after PS-liposomes capture are also physiopathological strategies that have already been described for tumour cells to escape immunosurveillance mechanisms. One of the most renowned is the secretion of VEGFA by tumour cells, which may be released not only to enhance angiogenesis and vascular permeability in order to promote tumour expansion, but also to preserve DCs in their immature state and prevent local inflammation (Yang *et al.*, 2018). Therefore, tumour cells rely on mechanisms that belong primarily to apoptotic cell clearance to induce immunological tolerance, and indeed, apoptotic mimicry-based liposomes might use the same pathways to achieve similar effects. In summary, this comparative transcriptome study has served to identify the specific gene signature of tolDCs generated by phagocytosis of PS-liposomes. This genomic program drives a particular behaviour in our tolDCs, which is different from that of iDCs and mDCs, and provides invaluable functionality-wise information rather than describe a simple state of maturation or lack thereof in these tolDCs.

We validated the RNA-seq results comparing the expression of 8 specific genes in iDCs and PSAB-DCs —namely, *CYTH4*, *GIMAP4*, *HPGD*, *NFKBIA*, *PLAUR*, *TNFAIP3*, *TNFSF14* and *VEGFA*—, but we also included the mDCs condition in order to assess the expression of these markers in DCs exposed to a pro-inflammatory stimulus. On the one hand, the *CYTH4* and the *TNFSF14* genes were apparently downregulated in mDCs in comparison to iDCs and PSAB-DCs, further supporting the idea that their upregulation provided DCs with tolerogenic features. On the other hand and to our surprise, genes that we had deemed as regulatory, such as *TNFAIP3*, *NFKBIA* and *VEGFA*, showed a trend to be expressed at higher levels in mDCs than in iDCs and PSAB-DCs. In this sense, other authors have reported that DCs matured with TNF-α display an upregulation of the immunoregulatory *NFKBIA* and *IDO* genes, and that maturation of DCs with a CC containing TNF-α, IL-1β and PGE_2 increases the transcription of regulators such as *NFKBIA*, *TNFAIP3* and *IDO* (Schinnerling *et al.*, 2015). These previous reports shed light on our results, and made us realise that it is ill-defined to try to correlate the functionality of the DCs, tolerogenic or otherwise, to the expression of a single gene or a handful of genes. In fact, the RNA-seq results informed us that at least 233 genes need to be differentially expressed for a DC to be rendered tolerogenic after the engulfment of PSAB-liposomes. Furthermore, it is widely accepted that each protocol used for tolDCs generation targets distinct signalling pathways to reach a tolerogenic functionality (Navarro-Barriuso *et al.*, 2018b), and

that, in the immune system, many molecules and genes exist to which a dual role has been assigned. Perhaps, the closest example is PGE_2, which *moonlights* as an immunoregulatory molecule in low amounts and as a powerful pro-inflammatory agent in high amounts (Kalinski, 2012).

It is worth mentioning that one of the hurdles in the implementation of tolerogenic cell-therapies has been none other than the heterogeneity of the *ex vivo*-generated tolDCs. To date, a common signature of tolerogenicity has been impossible to define, probably due to individual differences in human DCs and the infinite amount of agents and protocols used in the manufacturing of tolDCs. Work is currently in progress to define and standardise a set of transcriptional, phenotypical and functional characteristics of tolDCs (Lord *et al.*, 2016), which would serve to guarantee the safety of the product before administration and to compare the results between different manufacturing protocols. So far, tolDCs are accepted as reluctant to maturation and stable in a state of low expression of antigen-presenting and costimulatory molecules, along with a tolerogenic-skewed cytokine profile (ten Brinke *et al.*, 2015). These biomarkers are robust and provide reliable information about the functionality of the cell product, but certain drawbacks need be overcome: e.g., the phenotypical characterisation by flow cytometry inherently possesses high variability, and functional studies may require several days to be performed (Navarro-Barriuso *et al.*, 2018b). Perhaps, the definition of a catalogue of DEGs specifically involved in the tolerogenic functionality of tolDCs would emerge as an invaluable quality control of their proper generation, safety and functionality, but the fact remains that the transcriptomic profile of tolDCs strongly depends on the approach used to generate them. In this sense, one of the advantages of the direct administration of PS-liposomes would be the generation of tolDCs *in vivo*, and the subsequent avoidance of *ex vivo* cell manipulation and the required tolerogenic assessment. Indeed, our previous results in mice support this hypothesis (Pujol-Autonell *et al.*, 2015, 2017). Also, our RNA-seq analysis showed that, despite the heterogenous transcriptomics of DCs in basal conditions of the eight patients, the changes in gene expression after PS-liposomes phagocytosis were skewed towards a similar profile.

Therefore, the unveiling of this picture of tolerance induction in terms of transcriptomic, phenotypic and functional changes in DCs warranted further efforts in defining the potential of PS-liposomes to tackle autoimmune diseases, and T1D in particular. In fact, T1D was a lethal condition no more than one hundred years ago, but the discovery of the insulin hormone and its mass-production has allowed patients to live longer and has dramatically improved their quality of life. Yet, exogenous insulin replacement therapy does not cure T1D but converts it into a chronic disease. The existence of insulin does not render its management into an easy task either since, briefly, it entails constant monitoring of glycaemia levels, calculation of ingested car-

bohydrates, and adjustment of insulin doses considering the amount and nature of the carbohydrates, exercise performance and fluctuating insulin sensitivity. If more insulin than necessary is administered, consequent hypoglycaemia may leave the patient unable to function normally; if injected insulin is insufficient, continuous hyperglycaemia may lead to the development of long-term secondary complications, such as retinopathy, nephropathy or cardiovascular conditions (Melendez-Ramirez *et al.*, 2010). Finding the optimum balance is very demanding on the patients when they try to manage the disease themselves, but it is even more complicated to achieve when parents have to control the diabetes of their children (Streisand and Monaghan, 2014). Moreover, diabetes represents a huge economic impact on the national health system. Knowing that T1D incidence in childhood is currently increasing (Patterson *et al.*, 2019), that its course is more aggressive and that it entails additional complications in its management, it was crucial for us to test the effects of our PS-liposomes therapy in DCs from children diagnosed with T1D.

Thus, we reached for the Paediatrics section of the Germans Trias i Pujol Hospital in order to recruit paediatric patients with T1D. Contrary to the study performed with adult cells, we decided to include patients at onset as well as with established disease in order to determine if the DCs from the two groups behaved similarly in terms of phagocytic activity and tolerogenic potential. To that aim, the patients were recruited, and we looked for control subjects that were age-matched and sex-matched to the patients at the onset, since we reasoned that patients at the onset would be the most differing. When comparing the clinical data of the groups, we found that patients with the established disease were older than the other two groups, which can be explained by the similar age of T1D diagnosis of the two groups of patients and the premise that patients with established disease had been diagnosed, at least, six months before. Similarly, patients with established disease had higher BMI than the other two groups, which was expected according to the distribution of BMI in relation to age (Swanton *et al.*, 2017), but nonetheless healthy in order to avoid the hypothetical interference of inflammatory processes associated with overweight and obesity (Monteiro and Azevedo, 2010). Also, patients with established disease had lower HbA1c levels in comparison to patients at onset. This was expected since the former had at least six months of insulin replacement therapy, allowing for an improved control of hyperglycaemia, and the latter were recruited in the same week of diagnosis. Finally, and also as described for young patients (Davis *et al.*, 2015), fasting C-peptide levels were undetectable in the majority of patients with established disease.

Once the patients were recruited, we were faced with the challenge of obtaining monocytes from 10 mL of peripheral blood samples, which was the maximum amount that the corresponding Ethical Committee allowed to be drawn from children. In our hands, it was indeed complex to magnetically isolate monocytes from PBMCs in the

same manner that we did from adult samples, and so we opted to magnetically isolate monocytes directly from peripheral blood. Albeit the small number of monocytes yielded, purity of $CD14^+$ cells and viability were optimum. Strikingly, patients with established disease had similar CD14 purity in the isolated samples to patients at the onset, but lower when compared to control subjects, perhaps due to sample handling. Notwithstanding, we monitored the acquisition of the CD11c marker as means to control the efficiency of differentiation of the monocytes into DCs, and confirmed that it was alike in the three groups, indicating that DCs could be optimally generated with this method as well.

Subsequently, we tested the effect of PS-liposomes on the viability of paediatric DCs, which did not reveal any toxicity. Afterwards, we discovered that DCs from patients with established disease captured PS-liposomes more slowly and less efficiently than DCs from control subjects, and that DCs from patients at onset did also phagocyte PS-liposomes more slowly than those from control subjects. Since the value of AUC correlated negatively only with the time of disease evolution, we hypothesised that the phagocytic activity of DCs could be impaired as the disease progresses, perhaps due to a temporal accumulation of dysglycaemia. Other authors have reported, for instance, that the phagocytic activity of macrophages in patients with T2D is reduced when the glycaemic control is not optimal, but that it can be recovered when metabolic control is improved (Lecube *et al.*, 2011). Similarly, the tolerogenic functionality of DCs in patients with T1D is strengthened when the control of glycaemia is improved (Dáňová *et al.*, 2017). In our set of patients, however, the disease is better managed by patients with established disease judging by the HbA1c values, and so the conclusion that only glycaemia control can determine the phagocytic activity of these DCs cannot be drawn. In this regard, other examples in the literature describe that the phagocytic activity of macrophages is altered in other settings where glucose metabolism is not a key player. As a case in point, macrophages from patients with systemic lupus erythematosus show a reduced capacity for apoptotic cell clearance *in vitro* (Herrmann *et al.*, 1998). Furthermore, patients with chronic obstructive pulmonary disease and cystic fibrosis exhibit the same impairment (Hodge *et al.*, 2003; Vandivier *et al.*, 2002), and even smoking has been reported to inhibit actin rearrangement in phagocytes, impairing the ability of macrophages to engulf apoptotic cells (Minematsu *et al.*, 2011).

Interestingly, macrophages from the NOD mouse model also display a reduced capacity to efferocyte apoptotic cells, both at early ages when diabetes has not yet developed and at adulthood, in comparison to other strains (O'Brien *et al.*, 2002). The authors also reported that NOD mice's peritoneal macrophages were as efficient as other strains' were in the capture of PS-free microspheres, which suggested that the impaired phagocytic activity was specific for apoptotic cells. Following this

train of thought, we assessed the phagocytosis kinetics of PC-liposomes by paediatric DCs from selected control subjects and patients at the onset. While we observed that the phagocytosis kinetics of PC-liposomes tended to be slower than the phagocytosis of PS-liposomes in these subjects —as it was in adult cells— the results also made apparent that DCs from patients at onset phagocyted PC-liposomes in an even more slowly manner than their counterparts from control subjects. Taken together, these data suggest that several factors, which may include intrinsic defects, dysregulation of signalling pathways, dysglycaemia and actin rearrangement, could contribute to the impairment of phagocytosis in paediatric T1D. Finally, we considered whether these results could be due to the different techniques employed to isolate monocytes from paediatric or adult blood samples. However, when comparing the phagocytosis kinetics of DCs from adult and paediatric control subjects, no differences were found, and reached the 80% efficiency of PS-liposomes capture mark after 4 hours of coculture equally. Thus, we ruled out this hypothesis and continued searching for a reasonable explanation to these differences.

Since our work is focused on PS-liposomes, we started on assessing the expression of PS receptors in DCs from paediatric subjects. In a rather straightforward hypothesis, we theorised that one of them would be expressed at different levels between the three groups and thus would explain the different kinetics of PS-liposomes phagocytosis. To our surprise, while DCs from patients at onset showed a tendency to express *CD36* at lower levels and *MFGE8* at higher levels than the other groups, DCs from patients with established disease showed a trend for higher expression of *CD68* and *MERTK* genes and a significantly lower expression of *MFGE8*. As aforementioned, it is widely understood that the different PS receptors work synergistically to internalise apoptotic cells (Moller-Tank and Maury, 2014) and, in the same manner, PS-containing vesicles. For example, the protein MerTK induces the capture of apoptotic cells in macrophages, but it requires a previous attachment of PS by the protein TIM-4 due to its higher binding affinity to PS (Nishi *et al.*, 2014). Similarly, MerTK can work synergistically with integrin αvβ5 (Wu *et al.*, 2005), and TIM-4 and MFG-E8 are involved in the capture of apoptotic cells after the complex MFG-E8/integrin has signalled their uptake (Toda *et al.*, 2012). Thus, we cannot discard that the phagocytosis defect might be explained by the suboptimal expression or function of several PS receptors, or that we have yet to find the one responsible for such finding. On another note, very little information is known about the phagocytic activity of DCs, since the majority of studies in that regard have been conducted with macrophages. Thus, it is plausible that DCs may express the same PS receptors as macrophages but in different proportions, or express entirely different PS receptors (Trahtemberg and Mevorach, 2017), which could ultimately be accountable for the impaired phagocytosis of paediatric DCs.

Afterwards, we also studied the expression of two immunoregulatory genes downstream in the efferocytosis signalling pathway, *PPARG* and *TGFB1*. Interestingly, DCs from patients with established disease expressed higher levels of *PPARG* than those of other groups, and *TGFB1* showed a trend for higher expression in DCs from both groups of patients when compared to controls. These results suggest that, albeit their impairment in phagocytosis activity, DCs from paediatric patients with T1D could make attempts at immunoregulation through different signalling pathways. Indeed, coculture with PSAB-liposomes for 4 hours seemed to induce a slight upregulation of *TGFB1* expression, both in DCs from patients at onset and with established disease but not from control subjects, and *MERTK* gene showed a tendency to be upregulated after PSAB-liposomes phagocytosis in DCs from patients with established disease and control subjects. In this sense, MerTK, aside from a known PS receptor, is a marker of human tolDCs and is responsible for the blockade of NF-κB activation after the capture of apoptotic cells, pro-inflammatory cytokine secretion and costimulatory molecule upregulation (Sen *et al.*, 2007; Wallet *et al.*, 2008). Thus, it is encouraging that PSAB-liposomes capture induced the upregulation of *TGFB1* and *MERTK* genes in DCs from patients with established disease, which show the most impaired ability to phagocyte PS-liposomes. In control subjects, however, the only apparent effects of PSAB-liposomes phagocytosis in DCs were the upregulation of *CD36* and *MERTK* genes, perhaps in order to provide a positive feedback for phagocytosis and to unfold an immunoregulatory response. It could be that efferocytosis, PS receptors' expression and consequent immunoregulation are modulated differently in DCs from control subjects, patients with T1D at onset or with established disease, and that it might compensate for other impaired molecules or pathways in such a way that the tolerance induction is ultimately optimum.

While on the subject of tolerogenic potential, we also determined the expression of tolerogenic markers in DCs from paediatric subjects which had been found upregulated in the RNA-seq performed with adult cells. First, DCs from both patients at onset and patients with established disease showed a trend for higher expression of *TNFAIP3* and *TNFSF14* than control subjects, further supporting the notion that DCs from patients make attempts at immunoregulation through different pathways. Interestingly, the expression of *Tnfaip3* in murine DCs has been found upregulated after apoptotic cell phagocytosis (Cummings *et al.*, 2016), and this expression reportedly decreases their rate of apoptotic cell clearance and prompts their acquisition of tolerogenic potential (Kool *et al.*, 2011). It is noticeable that DCs from all groups seemed to upregulate *TNFAIP3* gene expression after PSAB-liposomes phagocytosis. However, whereas the first two groups only show a minor trend, the *TNFAIP3* upregulation in DCs from patients with established disease almost reached statistical significance, and this could provide a plausible explanation for their im-

paired PS-liposomes phagocytosis. On the other hand, *TNFSF14* was upregulated after PSAB-liposomes phagocytosis only in DCs from patients at onset, perhaps in response to the upregulation of the *VEGFA* gene in the same group, as described (Petreaca *et al.*, 2008). As for the effect of PSAB-liposomes on the expression of *VEGFA*, it is interesting that only DCs from patients with T1D displayed an apparent *VEGFA* upregulation, which is reminiscent of the effect of PSAB-liposomes in the expression of *TGFB1* in DCs from paediatric patients. In this sense, it would prove relevant to assess whether the upregulation of the *VEGFA* and *TGFB1* genes is translated into a stronger secretion of both proteins. As previously hypothesised, the upregulation of genes encoding for anti-inflammatory molecules only in DCs from paediatric patients could reflect a continuous attempt at immunoregulation prompted by the ongoing autoimmune reaction (Seyfert-Margolis *et al.*, 2006). Finally, *LAIR1* gene tended towards a higher expression in DCs from patients with established disease. Interestingly, *Lair1* overexpression in murine macrophages has been linked to an inhibition of CH-particle phagocytosis (Yi *et al.*, 2018). This finding provides yet another explanation for the reduced capacity of PS-liposomes capture observed in DCs from paediatric patients with established disease, since PS-liposomes do contain CH in addition to PS. Nevertheless, the defect in the phagocytic activity of DCs from paediatric patients with T1D is probably due to multiple factors, and so many efforts will have to be further dedicated in order to draw the intricate picture of the phagocytosis impairment.

Moreover, we studied the membrane expression of PS receptors and immune-related molecules in DCs from paediatric patients with T1D in order to asses if the activation of tolerogenic signalling pathways was also translated to the DC membrane. As it happened to the PSAB-DCs of adult patients, PSAB-DCs from paediatric patients preserved a high expression of PS receptors, which decreased when exposed to maturation stimuli. Interestingly, no differences were found in the levels of expression of PS receptors in DCs between patients at onset and with established disease, suggesting that any alteration in the expression of these PS receptors observed at 4 hours could have been solved at 24 hours at the membrane level. As for the expression of immune-related molecules —namely HLA-ABC, HLA-DR, CD54, CD86, CD40, CCR2, CXCR4, TLR2 and CD14—, it is indeed encouraging that PSAB-DCs from paediatric patients display the same behaviour that PSAB-DCs from adult patients did in regards to iDCs and mDCs, thus indicating that their phenotype could be optimum and in agreement with tolerogenic potential. However, other functional markers that were useful to determine the activation of PSAB-DCs from adult subjects, such as CD25, CCR7 and DC-SIGN, cannot be employed in the same manner in DCs from paediatric subjects, since their expression levels did not change in response to the stimuli. Even if we cannot fathom any reason to explain

these findings, the overall picture indicates that DCs from paediatric patients with T1D, both at onset and with established disease, are capable of inducing the expression of immunoregulatory genes and molecules, hinting that their tolerogenic abilities could remain unaltered even if the disease progresses.

Finally, we have established that autoantigen-loaded PS-liposomes could play a significant role in the re-establishment of tolerance in T1D, but the ideal moment to administer such immunotherapy is not clear. Other autoimmune diseases like MS or systemic lupus erythematosus recur in flares or exacerbations followed by a period of remission, which is perfectly aligned with the normal development of an immune response, in which an effector response is rapidly mounted to destroy the dangerous antigen and restricted after it has been successfully eliminated. In the setting of autoimmune diseases, however, the antigen is ever-present, and so *flares* could reflect those moments when the autoimmune response is invigorated, whereas the *remissions* might be the result of a dampening or a better regulation of the immune response. Therefore, the ideal moment in which to administer any antigen-specific immunotherapy to patients suffering from clinically relapsing-remitting autoimmune diseases might be during the remission phase. However, T1D does not show any clinical manifestation of a relapsing-remitting behaviour, but prominent authors have indeed hypothesised that the immunological process at the root of T1D may be relapsing-remitting in nature (von Herrath *et al.*, 2007). Yet, since the noticeable outcome of this remitting phases would be a better metabolic control prompted by a better function or improved regeneration of β-cells —which are hardly achieved in the normal development of T1D—, very few times this remission would be translated into a clinical benefit. Notwithstanding, the most unequivocal evidence fuelling this theory is the existence of a spontaneous and transient PR phase. As aforementioned, immune factors such as a transient recovery of adaptive immune tolerance are thought to contribute to the PR stage (Fonolleda *et al.*, 2017), which lead to an improvement in the β-cell function, but no specific biomarkers have been found to echo such a state so far. Ideally, if such biomarkers existed, they could be equally monitored during the whole progression of the disease to identify those subsequent and milder remission phases, in which β-cell function is not as improved to warrant a better metabolic control but the autoimmune response is equally subdued, and thus would be more prone to accept the *immunomodulatory change*. In this sense, we have recently reported that certain peripheral blood immune subsets are altered during the early stages of T1D in children (Villalba *et al.*, 2019). For instance, activated Treg cells and regulatory B cells are increased during the first year of progression, and levels of plasmatic TGF-β are diminished during the PR phase. Nevertheless, since PS-liposomes would have to be captured by DCs *in vivo*, we also studied the percentages and numbers of different subsets of peripheral blood DCs in this work.

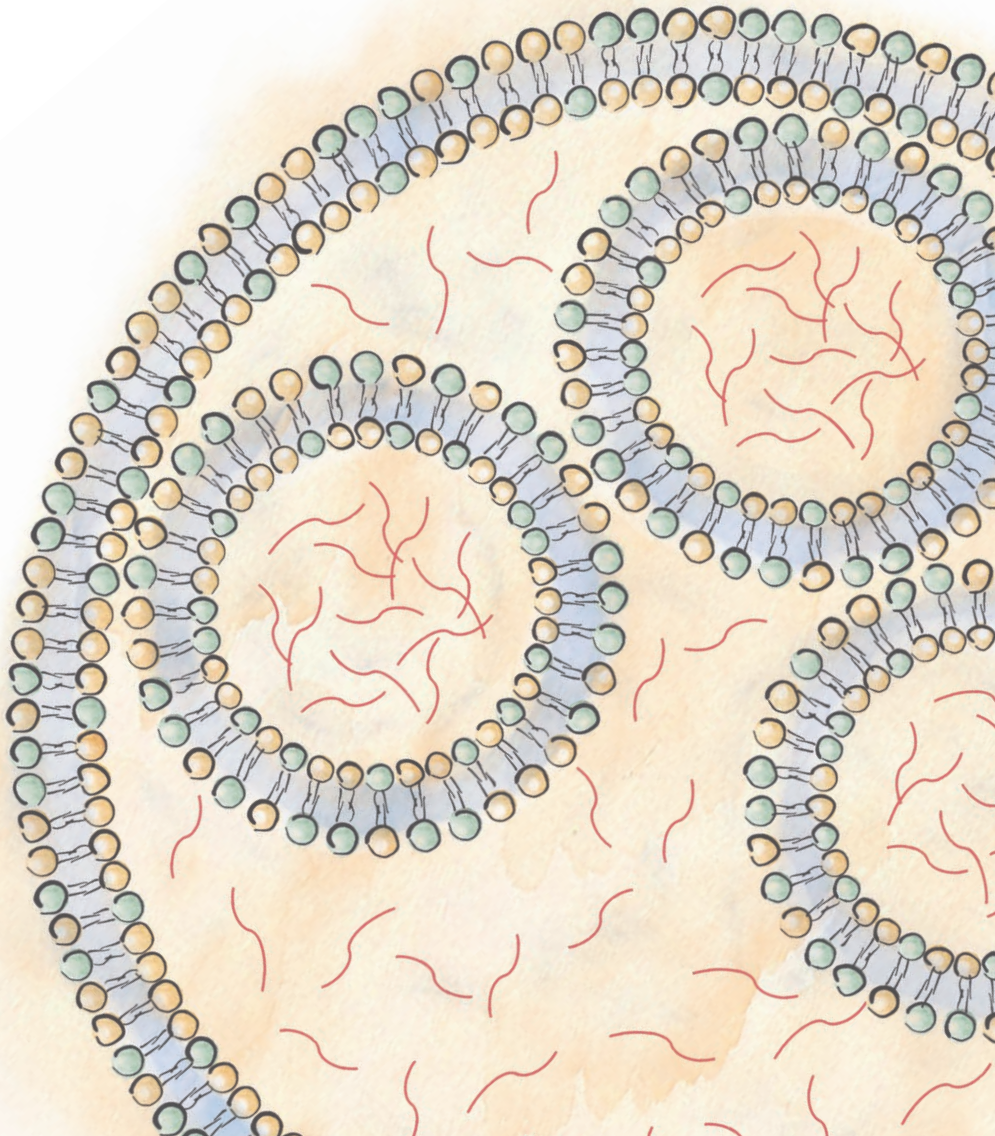
For this assessment, we used a different set of paediatric control subjects and patients with T1D, which were recruited at the onset and at the 1st and 2nd year of evolution. It is worth mentioning that the patients recruited at each time point are independent of those of other time points. Following the same reasoning used when comparing the clinical data of the previous set of patients, these patients were equally differing in the duration of the disease and the HbA1c values. Interestingly, patients at 1st year of evolution showed a trend to have the lowest HbA1c levels, the highest C-peptide levels and the lowest calculated insulin doses, probably reflecting the improved β-cell function of the PR stage, which coincidentally takes place during the 1st year of evolution of the disease (Fonolleda *et al.*, 2017). Interestingly, it was during the 1st year that we detected most of the alterations in the different peripheral blood DC subsets studied. As a case in point, during the 1st year, the percentage of myDCs and pDCs was increased in comparison to other time points, whereas both the percentage and numbers of the $CD11c^{-}CD123^{-}$ subset were decreased. Furthermore, when we divided the set of patients at 1st year depending on whether they were undergoing PR, the numbers of the $CD11c^{-}CD123^{-}$ subset displayed a slight tendency to be decreased in those patients experiencing PR. Future studies in which we follow the same patients during these time points may provide further insight into the biological significance of these results. In this sense, very few studies exist analysing the peripheral subsets of DCs in adults (Peng *et al.*, 2003) or children (Nieminen *et al.*, 2012; Vuckovic *et al.*, 2007) with T1D. For instance, these authors report a decrease in myDCs in children with T1D at recent onset, a finding that we were unable to identify perhaps due to differences in the marking of myDCs or the small sample size used in our study. Indeed, further efforts are warranted to validate the usefulness of the identification of peripheral blood DC subsets, as well as their use as biomarkers in the progression of the disease and in the marking of the ideal moment in which to administer an immunotherapy. Moreover, it would prove most interesting to assess the ability of peripheral blood DCs to phagocyte PS-liposomes, the kinetics in which they do so and the effects PS-liposomes elicit in the different subsets. Finally, we could identify and screen other new biomarkers that reflect the attempts at immunoregulation. Afterwards, these biomarkers could be used in the clinical practice to monitor the progression of T1D, and ideally, to stratify patients prior to clinical intervention and to characterise the immunomodulatory beneficial effect of any immunotherapy.

What is certain is that the re-establishment of tolerance to specific autoantigens will not be enough for the majority of patients, and so they will have to be coupled with regeneration strategies for the targeted tissue to recover its functionality. Therefore, immunotherapies able to re-educate solely the misguided autoimmune response are of highest priority, and autoantigen-loaded PS-liposomes constitute an

ideal candidate in such an endeavour. On the one hand, they rely on the inherent ability of apoptotic cells to call for the physiological tolerance-inducing mechanism known as efferocytosis, merely by being composed of a lipid mixture and PS, having a diameter greater than 500 nm and a negative surface charge. Also, they can encapsulate a wide range of autoantigenic peptides to direct the tolerance re-establishing process, without any difficulties in maintaining such physicochemical features, and so they can be potentially used as a platform to tackle several autoimmune reactions. On the other hand, they have the advantage of being easily manufactured and large-scale produced in a cost-competitive manner, customisable, standardisable and easy to administer. Most importantly, autoantigen-loaded PS-liposomes have arrested the autoimmune attack and re-established immunological tolerance through the generation of tolDCs and Treg lymphocytes in two experimental autoimmune diseases, and have proved effective in the generation of tolDCs in human, thus demonstrating their translational potential. Hence, the work herein reported validates PS-liposomes as a powerful tool to deliver a binary signal of tolerance and specificity, and achieves apoptotic mimicry in a synergistic, simple, safe and efficient manner. As encouraging as these results truly are, we cannot ignore the possibility that the autoantigen-loaded PS-liposomes immunotherapy might not work as expected once the clinics setting is reached. Indeed, further research and optimisation are imperative, and this work has but opened the door to do so. The extensive murine and human knowledge gathered so far and the future studies will enable the further improvement of the PS-liposomes immunotherapy and pave an even smoother way to its future clinical implementation. With their use, our ultimate goal is to contribute to lightening the burden that the diagnosis of an autoimmune disease entails in the current scenario.

In summary, with this work, we have contributed to unveil a picture of tolerance induction based on apoptosis mimicry in the setting of autoantigen-driven autoimmune diseases. We have studied the transcriptomic, phenotypic and functional changes that PS-liposomes phagocytosis induces in DCs, which provides them with such tolerogenic potential that they are able to arrest autoimmune aggression. While still *en route* to translationality, our results bring this biocompatible immunotherapy even closer to a clinical setting, and encourage us to delve deeper into the mechanisms by which PS-liposomes achieve tolerance re-establishment. Indeed, we are confident that, in the near future, the immunotherapeutic intervention aiming to restore immunological tolerance through apoptotic mimicry with the use of unsophisticated but clever PS-liposomes could prove revolutionary in the field of antigen-specific autoimmune disorders.

CONCLUDING REMARKS



1. Apoptotic cell-mimicking PS-liposomes can be adapted to different autoimmune diseases, as demonstrated by the efficient encapsulation of relevant autoantigenic peptides into PS-liposomes with optimum diameter and surface charge.
2. Contributing to the long-term re-establishment of tolerance in the T1D model, the administration of autoantigen-loaded PS-liposomes to NOD mice causes the expansion of cognate non-classical $CD4^+$ Treg cells and antigen-specific $CD8^+$ T cells *in vivo*.
3. The final PS-liposomes product is non-toxic for the NOD model, since severe posology and administration patterns are well-tolerated by NOD mice.
4. The potential of autoantigen-loaded PS-liposomes to be used as a platform for the tackling of autoimmune diseases is validated, as it is effective in re-establishing tolerance in experimental MS as well as T1D. In the EAE-induced model, PS-liposomes elicit tolerogenic characteristics in DCs and cause a reduction in its incidence and severity correlating with an increase in non-classical $CD4^+$ Treg cells.
5. The effect of autoantigen-loaded PS-liposomes in human DCs is similar to that observed in experimental models, and is suitable for tolerance induction. The phagocytosis of autoantigen-loaded PS-liposomes by DCs from human adult patients with T1D is prompted by PS and elicits immunomodulatory features in terms of phenotype, functionality and gene expression.
6. The tolerogenic effect of autoantigen-loaded PS-liposomes in human DCs from paediatric patients with T1D is evident in terms of phenotype and expression of immunoregulatory genes. However, DCs from patients with established disease show defects in their phagocytic capacity correlating with the time of disease progression, albeit that does not hinder their tolerogenic potential.
7. The liposomal immunotherapy herein described, which is based on the physiological tolerance-inducing mechanism known as efferocytosis, achieves apoptotic mimicry in a simple, safe and efficient manner. Therefore, the PS-liposomes immunotherapy constitutes a promising strategy to arrest autoimmune aggression and restore immunological tolerance in several autoantigen-driven autoimmune diseases.

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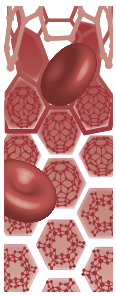
Research Article

Nanomedicine



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

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

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Keywords: autoimmunity • experimental autoimmune encephalomyelitis • immunotherapy • liposomes • multiple sclerosis

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

We recently developed a liposome-based immunotherapy – inspired by the features of apoptotic cells [7] – to re-establish tolerance to self and avoid autoimmune diseases [8]. These liposomes are rich in phosphatidylserine (PS), a phospholipid membrane component that is exposed in apoptotic cells and modulates immune responses [9], and are loaded with self-peptides – named autoantigens – as the selective molecule for tolerance induction. PS is recognized by membrane receptors of antigen presenting cells – mainly dendritic cells (DCs) – acting as an 'eat me' and 'tolerate me' signal, which allows the encapsulated autoantigen to be presented in a tolerogenic manner. Working synergistically, PS-liposomes and encapsulated autoantigens display a big translational potential in pathologies that require the re-establishment of immune tolerance.

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

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

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

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

Materials & methods

Mice

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

Liposomes & peptides

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

DC generation

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

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

Liposome capture by DCs

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

Assessment of DCs phenotype after liposome uptake

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

Prostaglandin E2 quantification

The production of prostaglandin E_2 (PGE_2), a potent immunoregulator responsible for the immunosuppressive mechanism of apoptotic cells, was assessed by ELISA (PGE_2 EIA Kit-Monoclonal; Cayman Chemicals, MI, USA), in supernatants obtained from DC cultures, 24 h after liposome capture. LOD: 80% B/B_0: 15 pg/ml. Sensitivity: 50% B/B_0: 50 pg/ml. Results were expressed as pg of $PGE_2/10^6$ cells.

Disease induction & clinical follow-up in the EAE model

For the induction of EAE, C57BL/6 female mice (Envigo) at 8 weeks of age received subcutaneous injections in both flanks of 50 µg $MOG_{40–55}$ peptide in PBS, emulsified in an equal volume of complete Freund's adjuvant, containing 4 mg/ml of Mycobacterium tuberculosis H37RA (Difco, MI, USA), under ketamine/xylazine at 50 and 5 mg/kg body weight, respectively. In addition, 250 ng of Pertussis toxin (Sigma) were injected intravenously at day 0 and 2. All animals were weighed and examined daily for welfare and clinical signs, according to the following criteria: 0, asymptomatic; 0.5, loss of distal half of tail tone; 1, loss of entire tail tone; 1.5, hind limb weakness; 2, hind limb paralysis; 2.5, hind limb paraplegia; 3, forelimb weakness; 4, quadriparesis; 4.5, severe quadriparesis; 5, quadriplegia; and 6, death. Clinical follow-up analyzes were performed in a blinded manner by two different observers.

Treatment of EAE with liposome-based immunotherapy

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

Analysis of regulatory T cells

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

Table 1. Liposome features.

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

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

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

Statistical analysis

The statistical analysis was performed using the Prism 6.0 software (GraphPad software Inc., CA, USA). For comparisons of unpaired data, a nonparametric Mann–Whitney test was used; for paired comparisons, a nonparametric Wilcoxon test was used. Fisher's exact test was used to compare qualitative variables. For correlation between parameters, Spearman's test was used. A p-value ≤0.05 was considered significant.

Results

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

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

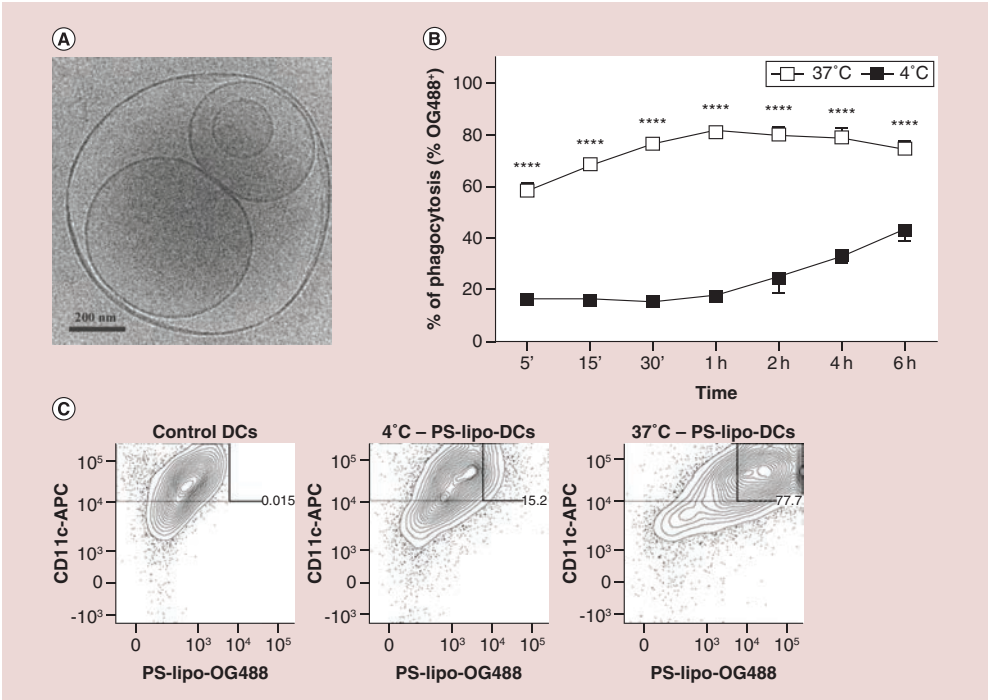

Figure 1. Liposomes are phagocytosed by dendritic cells. (A) Cryogenic transmission electron microscopy images of SMOG-liposomes. Bar = 0.2 μm. **(B)** Time course of the capture of PS-lipo-OG488 by DCs at 37°C (white squares) and at 4°C (black squares). Results are mean ± SD of three independent experiments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

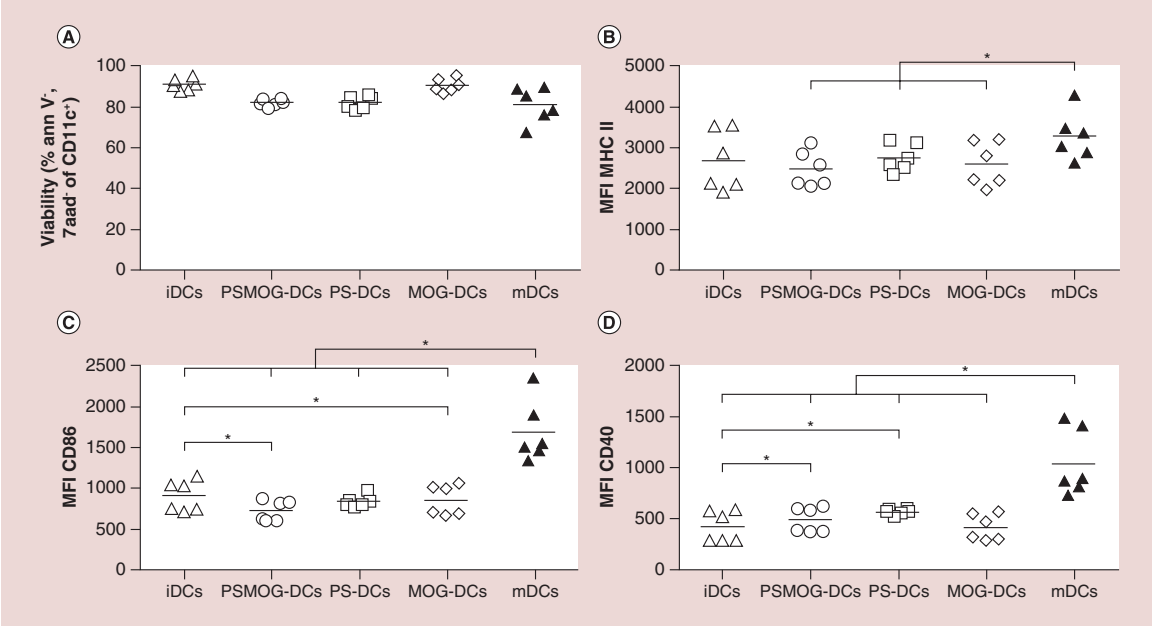

Figure 2. Effect of the capture of phosphatidylserine-liposomes in dendritic cells phenotype. (A) DCs viability assessed by annexin V and 7-AAD staining. White symbols represent iDCs alone (triangles) and after the capture of MOG peptide (MOG-DCs, rhombus), empty PS-liposomes (PS-DCs, squares) or MOG-filled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

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

PS-liposomes are efficiently phagocytosed by DCs

Time course analysis (Figure 1B) revealed that the maximum capture of PS-liposomes by DCs (81.53 ± 2.05%, mean ± SD) was achieved after 1 h of co-culture at 37°C. OG488 fluorescence signal at 37°C was higher ($p < 0.0001$) in each checkpoint when compared with its counterpart at 4°C. The significantly reduced OG488 signal in the experiments performed at 4°C as well as the large diameter of the vesicles (>500 nm) confirms the active process of phagocytosis by which liposomes are captured. Figure 1C shows a representative contour plot of PS-lipo-OG488 uptake by DCs after 30 min of co-culture at 4 and 37°C.

PS-liposomes preserve an immature phenotype in DCs

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

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

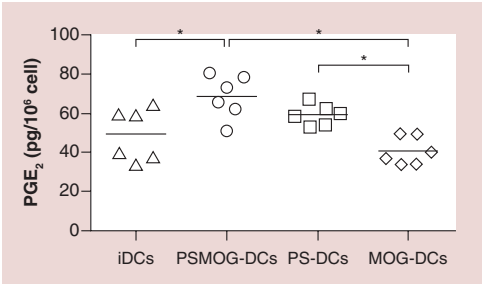

Figure 3. Dendritic cells produce PGE_2 after phagocytosis of PS-rich liposomes loaded with myelin-oligodendrocyte glycoprotein. Quantification of PGE_2 released by iDCs in culture medium (triangles), co-cultured with PSMOG-liposomes (circles), empty PS-liposomes (squares) or MOG peptide (rhombus) after 24 h of culture. Data are represented as pg/10^6 cells. Lines show the mean of six independent experiments. Comparisons between groups showed significant differences (*$p < 0.05$, nonparametric Wilcoxon test). DC: Dendritic cell; iDC: Immature DC; MOG: Myelin-oligodendrocyte glycoprotein peptide 40–55; PGE2: Prostaglandin E2; PS: Phosphatidylserine; PSMOG: PS-rich liposome loaded with myelin-oligodendrocyte glycoprotein.

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

Based on our previous results, PGE_2 production by DCs after PS-liposomes uptake was assessed in culture supernatants. The concentration of PGE_2 was significantly increased in PSMOG-DCs in comparison to iDCs and MOG-DCs ($p < 0.05$), as well as in PS-DCs when compared with MOG-DCs ($p < 0.05$) (Figure 3).

PS-liposomes filled with MOG peptides reduce EAE severity

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

PSMOG-liposomes effect on T-cell subtypes

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

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

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

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

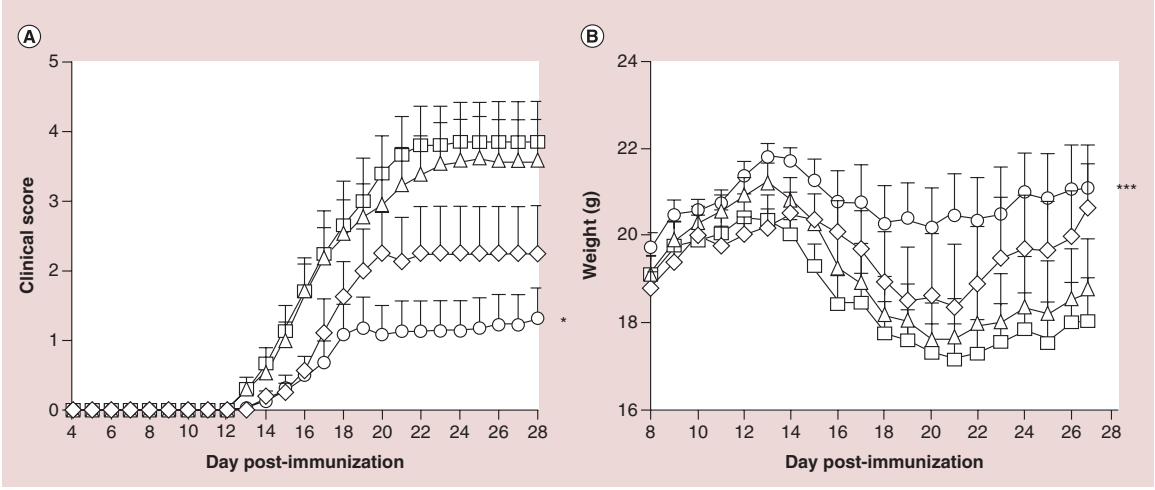

Figure 4. PS-rich liposomes loaded with myelin-oligodendrocyte glycoprotein prevent the development of experimental autoimmune encephalomyelitis. (A) Clinical score of experimental autoimmune encephalomyelitis performed daily for welfare and clinical status as well as neurological signs according to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

± 3.48% (mean ± SD) and 14.69 ± 2.67%, respectively ($p < 0.05$). However, we noted that the percentage of $CD25^+$ $FoxP3^-$ T cells in PSMOG-liposomes group increased when compared with their sham counterparts, that being of 14.83 ± 5.47% and 9.65 ± 2.84%, respectively ($p < 0.05$) (Figure 5A). Indeed, the percentage of $CD25^+$ $FoxP3^-$ T cells in PSMOG-liposomes treated mice correlated inversely with the clinical score at day 15 p.i. (Spearman's $r = -0.9258$, $p < 0.0001$), as shown in Figure 5B, but no correlation was observed between these parameters in sham group (data not shown). Nevertheless, the number of $CD25^+$ $Foxp3^+$ cells tended to decrease in PSMOG-liposomes group ($6.31 \times 10^5 \pm 2.03 \times 10^5$), when compared with sham group ($11.05 \times 10^5 \pm 0.76 \times 10^5$), although differences are not statistically significant. Regarding the absolute number of $CD25^+$ $FoxP3^-$ T cells, similar results were obtained in PSMOG-liposomes treated group ($9.13 \times 10^5 \pm 4.58 \times 10^5$) and sham group ($8.95 \times 10^5 \pm 1.71 \times 10^5$). With these results, the expression of $CD4^+$ Tr1 cell markers CD49b and LAG-3 was next examined on the splenocytes of the sham and PSMOG-liposomes groups by analyzing their median of fluorescence intensity. Although there were no statistically significant differences in the expression of CD49b between sham (90.18 ± 11.49, mean ± SD) and treated (95.12 ± 13.84) groups, a tendency for this marker to increase in PSMOG-liposomes treated mice was observed (Figure 5C). Supporting this tendency, LAG-3 expression was significantly increased in PSMOG-liposomes treated mice (387.5 ± 88.53) when compared with sham group (304.6 ± 54.41) ($p \leq 0.05$).

Discussion

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

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

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

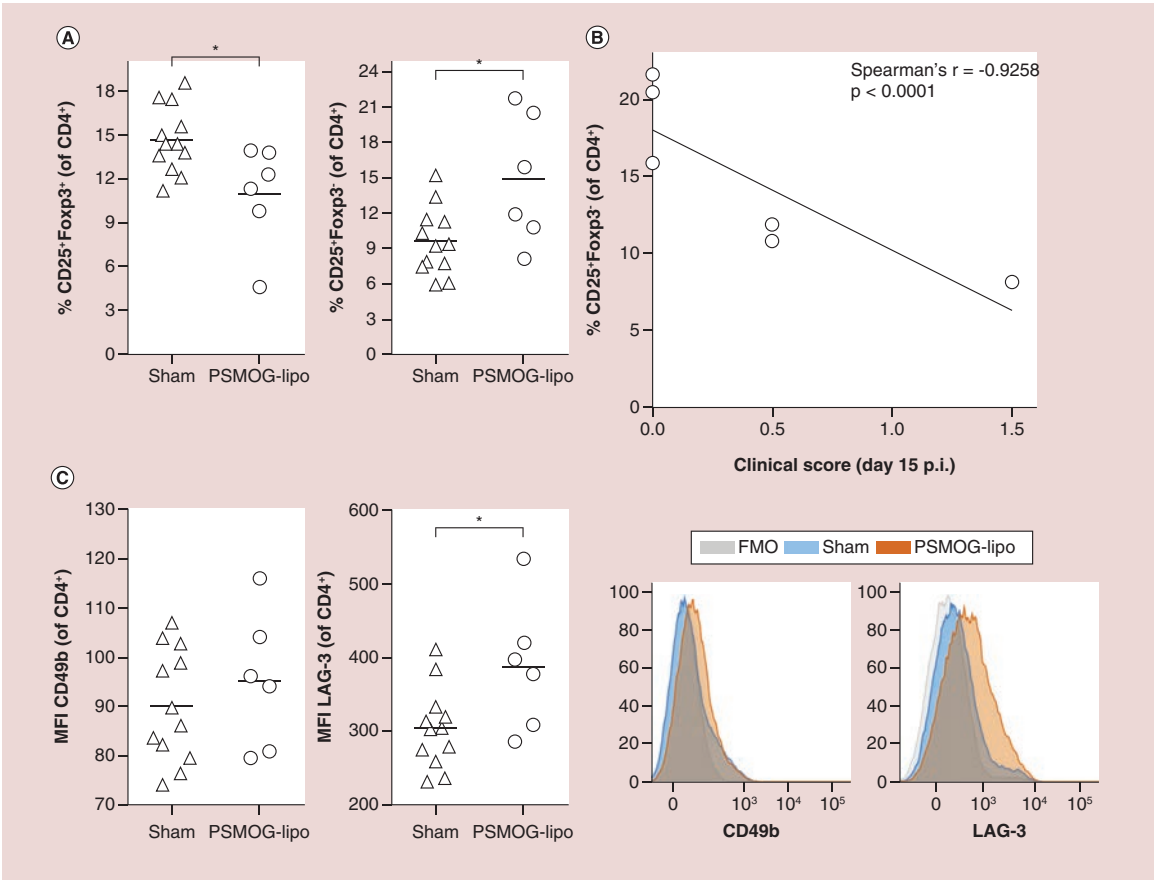

Figure 5. T cell subsets in mice treated with PS-rich liposomes loaded with myelin-oligodendrocyte glycoprotein. (A) Percentage of classical regulatory T cells ($CD25^+$ $FoxP3^+$ cells gated in $CD4^+$ T cells) and $CD25^+$ $FoxP3^-$ cells (gated in $CD4^+$ T cells) in the spleen of PSMOG-liposomes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

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

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

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

In vitro experiments revealed crucial immunological consequences of the capture of PSMOG-liposomes by iDCs. First, liposomes were rapidly, actively and safely captured through phagocytosis by DCs, as demonstrated by co-culture experiments at 37 and 4°C [38,39]. Endocytic processes involve the internalization of large-sized particles or a large volume of the extracellular bulk. These are termed phagocytosis and macropinocytosis, respectively: *phagocytosis* refers to the transport of large particles (>250 nm in diameter) by immune system specialized cells (macrophages, DCs and neutrophils), while *pinocytosis* refers to ingestion of fluids and solutes (about 100 nm in diameter) by mammalian cells [40]. In this sense, the large size of the here reported liposomes – diameter greater than 500 nm – and the presence of PS as an 'eat-me' signal promote active phagocytosis by DCs [8,41]. Second, after liposome engulfment, DCs increased CD40 membrane expression and decreased CD86, and maintained low membrane expression of MHC class II expression, features of tolerogenic DCs. Interestingly, PSMOG-liposomes uptake induced the secretion of PGE_2, an essential mediator in the maintenance of tolerance after efferocytosis [42]. These results are in agreement with our previous study, using specific PS-liposomes for T1D.

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

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

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

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

Conclusion

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

Supplementary data

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

Financial & competing interests disclosure

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Summary points

Background

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

Aim

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

Results

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

Conclusion

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

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval and have followed the principles outlined in the Declaration of Helsinki for animal experimental investigation.

Open access

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Papers of special note have been highlighted as: • of interest; •• of considerable interest

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Phosphatidylserine-Liposomes Promote Tolerogenic Features on Dendritic Cells in Human Type 1 Diabetes by Apoptotic Mimicry

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Type 1 diabetes (T1D) is a metabolic disease caused by the autoimmune destruction of insulin-producing β-cells. With its incidence increasing worldwide, to find a safe approach to permanently cease autoimmunity and allow β-cell recovery has become vital. Relying on the inherent ability of apoptotic cells to induce immunological tolerance, we demonstrated that liposomes mimicking apoptotic β-cells arrested autoimmunity to β-cells and prevented experimental T1D through tolerogenic dendritic cell (DC) generation. These liposomes contained phosphatidylserine (PS)—the main signal of the apoptotic cell membrane—and β-cell autoantigens. To move toward a clinical application, PS-liposomes with optimum size and composition for phagocytosis were loaded with human insulin peptides and tested on DCs from patients with T1D and control age-related subjects. PS accelerated phagocytosis of liposomes with a dynamic typical of apoptotic cell clearance, preserving DCs viability. After PS-liposomes phagocytosis, the expression pattern of molecules involved in efferocytosis, antigen presentation, immunoregulation, and activation in DCs concurred with a tolerogenic functionality, both in patients and control subjects. Furthermore, DCs exposed to PS-liposomes displayed decreased ability to stimulate autologous T cell proliferation. Moreover, transcriptional changes in DCs from patients with T1D after PS-liposomes phagocytosis pointed to an immunoregulatory profile. Bioinformatics analysis showed 233 differentially expressed genes. Genes involved in antigen presentation were downregulated, whereas genes pertaining to tolerogenic/anti-inflammatory pathways were mostly upregulated. In conclusion, PS-liposomes phagocytosis mimics efferocytosis and leads to phenotypic and functional changes in human DCs, which are accountable for tolerance induction. The herein reported results reinforce the potential of this novel immunotherapy to re-establish immunological tolerance, opening the door to new therapeutic approaches in the field of autoimmunity.

Keywords: immunotherapy, autoimmunity, human type 1 diabetes, liposomes, tolerance, dendritic cells

Abbreviations: DC, dendritic cell; PS, phosphatidylserine; T1D, type 1 diabetes.

INTRODUCTION

Type 1 diabetes (T1D) mellitus is a metabolic disease caused by loss of tolerance to self and consequent autoimmune destruction of insulin-producing pancreatic β-cells (1). When β-cell mass decreases significantly, the individual's endogenous production of insulin is no longer able to meet metabolic demands, leading to overt hyperglycemia. Upon diagnosis, patients with T1D require exogenous insulin administration, and although this treatment has allowed them to survive, long-term complications due to glycemic imbalances are bound to arise (2, 3). T1D usually appears during childhood or adolescence, and its incidence is increasing an average of 4% per year (4). Despite knowing that both genetic and environmental factors contribute to its development, triggering events remain elusive. The autoimmune attack against β-cells is led by a mild leukocytic infiltrate—insulitis—consisting of dendritic cells (DCs), macrophages, T and B lymphocytes, and natural killer cells, which gradually advance through the islets (5). The first islet-infiltrating cells are DCs (6), which orchestrate the loss of tolerance to β-cell autoantigens, insulin being a key autoantigen in human T1D (7).

The first step to revert T1D would be to arrest the pathological recognition of β-cell autoantigens. Many immunotherapies have prevented and even reverted T1D in animal models (8), but clinical trials have corroborated how challenging T1D prevention and reversion is, and most of them have been unsuccessful (9). In this scenario, the development of new therapies to halt autoimmunity in T1D has become an urgent biomedical matter. An ideal immunotherapy should restore tolerance to β-cells, avoiding systemic side effects, and allow islet regeneration. One of the most efficient physiological mechanisms for inducing tolerance is apoptosis, a form of programmed cell death lacking inflammation. The uptake of apoptotic cells by professional phagocytes such as macrophages and immature DCs (iDCs) is named efferocytosis (10). The exposure of "*eat-me*" signals on the apoptotic cell surface is what promotes their specific recognition and phagocytosis. Phosphatidylserine (PS), a phospholipid usually kept in the inner leaflet of the plasma membrane, is a relevant signal for efferocytosis. This molecule is recognized by multiple distinct receptors on antigen presenting cells, including members of the TIM family, Stabilin-2, integrins, CD36, CD68, among others, as well as by soluble receptors that in turn bind to membrane receptors (11). After the capture, the apoptotic cell is processed, thus prompting the release of anti-inflammatory signals and presentation of autoantigens in a tolerogenic manner by DCs (12). Failure of this mechanism, owing to an increase of apoptotic β-cells or defects in efferocytosis, contributes to the loss of tolerance to self in the context of T1D (13).

Our group demonstrated that DCs acquired a tolerogenic phenotype and functionality after engulfment of apoptotic β-cells, and that they prevented T1D when transferred to non-obese diabetic (NOD) mice (14, 15). However, since finding a substantial source of autologous apoptotic β-cells for T1D immunotherapy would be impossible, we conceived an immunotherapy based on biomimicry that consisted of liposomes—phospholipid bilayer vesicles—displaying PS in their surface and containing autoantigenic peptides, thus resembling apoptotic cells. Indeed, apoptotic mimicry performed by PS-liposomes successfully restored tolerance to β-cells in experimental autoimmune diabetes, preventing disease development and decreasing the severity of insulitis (16). Moreover, by only replacing the autoantigenic peptide encapsulated within PS-liposomes, we confirmed the potential of this immunotherapy to prevent and ameliorate experimental autoimmune encephalomyelitis, the experimental model of multiple sclerosis (17). In both cases, we demonstrated that phagocytosis of autoantigen-loaded PS-liposomes induced a tolerogenic phenotype and functionality in DCs, expansion of regulatory T cells and release of anti-inflammatory mediators that are responsible for arresting the autoimmune attack to target cells. Therefore, PS-liposomes could constitute a platform serving as a physiological and safe strategy to restore peripheral tolerance in antigen-specific autoimmune diseases. Liposomes, already used clinically as drugs deliverers for antitumor drugs and as vaccines (18), have the advantage of being safe and biocompatible, customizable, easily large-scale produced, and standardizable.

Aiming for the clinical potential of this strategy, we have encapsulated human insulin peptides to assess the effect of PS-liposomes in human DCs from patients with T1D and control subjects *in vitro*. We herein report that PS-liposomes are efficiently captured by human DCs, thus eliciting transcriptomic, phenotypic, and functional changes that point to tolerogenic potential. This immunotherapy constitutes a promising strategy to arrest autoimmune aggression in human T1D, benefiting from the co-delivery of tolerogenic signals and β-cell autoantigens.

MATERIALS AND METHODS

Patients

Patients with T1D ($n = 34$) and control subjects ($n = 24$) were included in this study. All patients with diabetes fulfilled the classification criteria for T1D. Inclusion criteria were 18–55 years of age, a body mass index (BMI) between 18.5 and 30 kg/m^2 and, for patients with T1D, an evolution of the disease longer than 6 months. Exclusion criteria were: being under immunosuppressive or anti-inflammatory treatment, or undergoing pregnancy or breastfeeding. For the RNA-sequencing (RNA-seq) experiment, we selected 8 patients of the 34 that participated in the study, but BMI was limited to a maximum of 24.9 kg/m^2, duration of the disease was restricted to a maximum of 5 years (in order to minimize the effect that long-term hyperglycemia could have on genetic and/or epigenetic profiles) and the presence of other chronic diseases became an exclusion criterion. All study participants gave informed consent, and the study was approved by the Committee on the Ethics of Research of the Germans Trias i Pujol Research Institute and Hospital.

Cell Separation and Generation of DCs

Peripheral blood mononuclear cells (PBMCs) were obtained from 50 ml blood samples of control subjects and patients with T1D by means of Ficoll Paque (GE Healthcare, Marlborough, MA, USA) density gradient centrifugation. Monocytes were further magnetically isolated using the EasySep Human CD14

Positive Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada) following the manufacturer's instructions. Once CD14 purity in the positively selected fraction was >70%, monocytes were cultured at a concentration of 10^6 cells/ml in X-VIVO 15 media (Lonza, Basel, Switzerland), supplemented with 2% male AB human serum (Biowest, Nuaillé, France), 100 IU/ml penicillin (Normon SA, Madrid, Spain), 100 µg/ml streptomycin (Laboratorio Reig Jofré, Sant Joan Despí, Spain), and 1,000 IU/ml IL-4 and 1,000 IU/ml GM-CSF (Prospec, Rehovot, Israel) to obtain monocyte-derived DCs. After 6 days of culture, DC differentiation yield was assessed by CD11c-APC staining (Immunotools, Friesoythe, Germany) and cell viability was determined by annexin V-PE (Immunotools) and 7aad staining (BD Biosciences, San Jose, CA, USA) using flow cytometry (FACS Canto II, BD Biosciences). The negatively selected fraction of PBMCs was cryopreserved in Fetal Bovine Serum (ThermoFisher Scientific, Waltham, MA, USA) with 10% dimethylsulfoxide (Sigma-Aldrich, Saint Louis, MO, USA) and stored for later use.

Peptide Selection and Preparation of Liposomes

Thinking in a future clinical application of liposomes, the two chains of insulin were selected to be encapsulated separately in order to avoid possible biological effects of insulin. A and B chains of insulin contain well-known β-cell specific target epitopes in human T1D (19). Peptide A corresponds to the whole human insulin A chain (21 aa, N-start-GIVEQCCTSICSLYQLENYCN-C-end), and peptide B is the whole human insulin B chain (30 aa, N-start-FVNQHLCGSHLVEALYLVCGERGFFYTPKT-C-end) (Genosphere Biotechnologies, Paris, France). Peptides were >95% pure and trifluoroacetic acid was removed. Liposomes consisted of 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (Lipoid, Steinhausen, Switzerland), 1,2-didodecanoyl-sn-glycero-3-phosphocholine (Lipoid), and cholesterol (Sigma-Aldrich). Liposomes were prepared using the thin film hydration method from a lipid mixture of 1,2-dioleoyl-sn-glycero-3-phospho-L-serine, 1,2-didodecanoyl-sn-glycero-3-phosphocholine and cholesterol at 1:1:1.33 molar ratio, respectively, as described (20). Liposomes without PS were generated as controls with 1,2-didodecanoyl-sn-glycero-3-phosphocholine and cholesterol at 1:1 molar ratio. All liposomes were produced under sterile conditions and at a final concentration of 30 mM. Lipids were dissolved in chloroform and the solvent was removed by evaporation under vacuum and nitrogen. The lipids were hydrated with the appropriate buffer (phosphate buffered saline or 0.5 mg/ml solution of peptide A or peptide B) and the liposomes obtained were homogenized to 1 µm by means of an extruder (Lipex Biomembranes Inc., Vancouver, BC, Canada). Peptide encapsulation efficiencies were calculated according to the equation: encapsulation efficiency (%) = $[(C_{peptide,total}-C_{peptide,out})/C_{peptide,total}] \times 100$, where $C_{peptide,total}$ is the initial peptide A or peptide B concentration and $C_{peptide,out}$ is the concentration of non-encapsulated peptide. To measure the $C_{peptide,out}$, liposome suspensions were centrifuged at 110,000 *g* at 10°C for 30 min. The concentration of non-encapsulated peptide was assessed in supernatants by PIERCE BCA protein assay kit (ThermoFisher Scientific). In addition to PS-rich liposomes loaded with insulin peptides [PSA-liposomes ($n = 3$) and PSB-liposomes ($n = 3$) encapsulating peptide A or peptide B, respectively], fluorescent-labeled liposomes with PS (empty fluorescent PS-liposomes, $n = 4$) and without PS (empty fluorescent PC-liposomes, $n = 4$) were also prepared using lipid-conjugated fluorescent dye Oregon Green 488 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Invitrogen, Carlsbad, CA, USA) and following the aforementioned methodology. Particle size distributions and stability—expressed as zeta potential (ζ)—were measured by dynamic light scattering using Malvern Zetasizer (Malvern Instruments, Malvern, UK) in undiluted samples. Liposome morphology and lamellarity were examined by cryogenic transmission electron microscopy (cryo-TEM) in a JEOL-JEM 1400 microscope (Jeol Ltd., Tokyo, Japan).

Phagocytosis Assay

To assess whether DCs were able to phagocyte liposomes, DCs were co-cultured with 100 µM of empty fluorescent PS-liposomes ($n = 5$ for control subjects and $n = 10$ for patients with T1D) or empty fluorescent PC-liposomes ($n = 6$ for control subjects and $n = 9$ for patients with T1D) at 37°C from 5 min to 24 h. As control, the same assay was performed at 4°C to confirm that liposomes were captured by an active mechanism of phagocytosis. Cells were extensively washed in cold phosphate buffered saline to remove all liposomes attached to the cell membrane. Liposome uptake was determined by flow cytometry (FACSCanto II, BD Biosciences).

Assessment of DCs Phenotype after Liposome Uptake

Although insulin chains were encapsulated separately, DCs were stimulated by a mixture of PSA-liposomes (50%) and PSB-liposomes (50%), in order to assess the effect of the whole insulin molecule as autoantigen. Thus, DCs from control subjects ($n \geq 5$) and patients with T1D ($n \geq 8$) were co-cultured with 1 mM of liposomes (PSAB-DCs) for 24 h in the presence of 20 µg/ml human insulin (Sigma-Aldrich), and their viability and phenotype were analyzed by flow cytometry (FACSCanto II, BD Biosciences). The sample number stated (n) is referred to the minimum number of control subjects and patients included in each experiment. As controls, DCs were either cultured with 20 µg/ml human insulin (Sigma-Aldrich) to obtain iDCs or adding a cytokine cocktail (CC) consisting of tumor necrosis factor (TNF)α (1,000 IU/ml, Immunotools), IL-1β (2,000 IU/ml, Immunotools) and Prostaglandin E_2 (PGE_2, 1 µM, Cayman Chemical, Ann Arbor, MI, USA) for 24 h to obtain mature DCs (mDC). Moreover, PSAB-DCs were cultured after phagocytosis with CC for 24 h in order to assess the response in front a pro-inflammatory stimulus (mPSAB-DCs). Phenotyping was performed as follows: DCs were stained with 7aad (BD Biosciences) and monoclonal antibodies to CD11c-APC, CD25-PE, CD86-FITC, HLA class I-FITC, HLA class II-FITC, CD14-PE and CD40-APC (Immunotools), CD36-APCCy7, TIM4-APC, αvβ5 integrin-PE, CD54-PECy7, TLR2-FITC, CXCR4-APCCy7, CCR2-APC, DC-SIGN-APC (Biolegend, San Diego, CA, USA) and CCR7-PECy7 (BD Biosciences). Corresponding fluorescence minus one staining was used as control. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

T Cell Proliferation Assays

Autologous T lymphocyte proliferation ($n = 12$ for control subjects and $n \geq 12$ for patients with T1D) was assessed by exposing PBMCs to the different conditions of DCs used in this study. Briefly, PBMCs from the same donor were thawed and stained with 0.31 μM CellTrace Violet (21) (ThermoFisher Scientific) according to manufacturer's instructions. The PBMCs were then co-cultured with iDCs, PSAB-DCs, mPSAB-DCs or mDCs at a 10:1 ratio (10^5 PBMCs:10^4 DCs). For each donor, 10^5 PBMCs were cultured in basal conditions as a negative control or with Phorbol 12-Myristate 13-Acetate (50 ng/ml, Sigma-Aldrich) and Ionomycin (500 ng/ml, Sigma-Aldrich) as a positive control. After 6 days of co-culture, proliferation was assessed in the different T cell subsets with CD3-PE, CD4-APC and CD8-FITC staining (Immunotools) by flow cytometry (FACS LSR Fortessa, BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.).

Cytokine Production

The Human Th1/Th2/Th17 kit (CBA system; BD Biosciences) was used to assess cytokine production. Culture supernatants from DCs and from T cell proliferation assays ($n \geq 3$ for control subjects and $n \geq 3$ for patients with T1D) were collected and frozen at −80°C until use. IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10 were measured. Data were analyzed using CBA software. The production of Human TGF-β1 by DCs after PSAB-liposome uptake was determined by ELISA (eBioscience, San Diego, CA, USA).

RNA-Seq of DCs before and after Liposome Phagocytosis

Dendritic cells obtained from patients with T1D ($n = 8$) were cultured in basal conditions (iDCs) or with 1 mM of PSA-liposomes and PSB-liposomes (PSAB-DCs) at 37°C for 4 h. Cells were then harvested from culture wells using Accutase (eBioscience), and viability and DC purity were assessed with 7aad (BD Biosciences), annexin V-PE and CD11c-APC (Immunotools) staining by flow cytometry (FACS Canto II, BD Biosciences). Liposome capture control assays were performed for every sample (see above *Phagocytosis Assay* section) to confirm phagocytosis. Supernatant was removed and cell pellets were stored at −80°C until use. RNA was extracted using RNeasy Micro Kit (QIAGEN, Hilden, Germany) and following manufacturer's instructions. RNA purity, integrity and concentration were determined by NanoDrop (ND-1000 Spectrophotometer, ThermoFisher Scientific) and 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). Afterward, 1 μg of total RNA was used to prepare RNA libraries following the instructions of the NebNext Ultra Directional RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). Library quality controls were assessed using a TapeStation 2200 (Agilent High Sensitivity Screen Tape) and a narrow distribution with a peak size of approximately 300 bp was observed in all cases. Libraries were quantified by qPCR using a QC KAPA kit (Hoffman-LaRoche, Basel, Switzerland) sequenced in a NextSeq 500 genetic analyzer (SBS-based sequencing technology, Illumina, San Diego, CA, USA) in a run of 2 × 75 cycles and a high output sequencing mode. Twenty million reads were obtained and analyzed for each sample. Fastq files coming from Illumina platform were merged and basic quality controls were performed with FASTQC and PRINSEQ tools. Paired-end (forward-reverse) sample merging was carried out with software CLCBio Genomics Workbench® version 8.5 (22), following the RNA-seq analysis pipeline found in CLCBio manuals. Read alignment and mapping steps to only gene regions were performed using CLCBio software against the human genome (Homo sapiens GRCh38 assembly, at both gene- and transcript-level tracks). The same software, with default options, was used to normalize counts by applying standard "Reads Per Kilobase of transcript per Million reads mapped" method. The remaining steps of the analysis were carried out with scripts and pipelines implemented with R software (23). The selection of differentially expressed genes (DEGs) was performed using the linear model approach implemented in the limma Bioconductor package (24), with previous log2-transformation of the normalized data. Adjusted p values of ≤0.125, taking into account multiple testing with the False Discovery Rate method, were considered significant. Therefore, genes with a p value <0.0013 and Log_2 of fold change >0.05 were considered upregulated, whereas those with Log_2 of fold change <0.05 were considered downregulated. Experimental data have been uploaded into European Nucleotide Archive (EBI, https://www.ebi.ac.uk/ena; accession number: PRJEB22240). DEGs were categorized using Ingenuity Pathway Analysis Software (QIAGEN), Protein Analysis Through Evolutionary Relationships Classification System (25), REACTOME Pathway database (26) and Gene Ontology Biological Process database (27). Furthermore, R software (23) was used to generate a gene heatmap of DEGs.

Quantitative RT-PCR

To validate transcriptome results, DCs obtained from patients with T1D ($n \geq 4$) and control subjects ($n \geq 3$) were cultured and pelleted in three conditions: iDCs, PSAB-DCs and mDCs. RNA was isolated using RNeasy Micro Kit (QIAGEN), and was reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). cDNA synthesis reactions were carried out using random hexamers (0.5 mg/ml, BioTools, Valle de Tobalina, Madrid, Spain) and reverse transcriptase Moloney-murine-Leukemia-virus (200 U/ml, Promega, Madison, WI, USA). Quantitative RT-PCR assays were performed with TaqMan universal assay (ThermoFisher Scientific) on a LightCycler® 480 (Roche, Mannheim, Germany) using the following TaqMan assays: *CYTH4* (Hs01047905_m1), *GIMAP4* (Hs01032964_m1), *HPGD* (Hs00960590_m1), NFKB inhibitor alpha (*NFKBIA*) (Hs00153283_m1), *PLAUR* (Hs00958880_m1), *TNFAIP3* (Hs00234713_m1), tumor necrosis factor superfamily member 14 (*TNFSF14*) (Hs00542477_m1), and *VEGFA* (Hs00900055_m1). Relative quantification was performed by normalizing the expression for each gene of interest to that of the housekeeping gene *GAPDH* (Hs02758991_g1), as described in the $2^{-\Delta Ct}$ method (28).

Statistical Analysis

The statistical analysis was performed using Prism 7.0 software (GraphPad software Inc., San Diego, CA, USA). Analysis of

variance (ANOVA) was used for comparisons with several factors. For comparisons of unpaired data, a non-parametric Mann-Whitney test was used; for paired comparisons, a non-parametric Wilcoxon test was used. A p value ≤ 0.05 was considered significant.

RESULTS

Patients with T1D and Control Subjects Display Similar Features

Thirty-four patients with T1D (50% female, 50% male) from the Germans Trias i Pujol Hospital and 24 control subjects (45.8% female, 54.2% male) met the inclusion and exclusion criteria and were included in the study (**Table 1**). Age of control subjects was 30.46 ± 8.18 years (mean ± SD), while that of patients with T1D was 32.54 ± 8.96 years; BMI was 23.90 ± 2.87 kg/m^2 and 23.80 ± 3.11 kg/m^2, respectively. Patients with T1D had been diagnosed at 20.79 ± 9.90 years, had a duration of disease of 11.75 ± 9.70 years, and a hemoglobin A1c level of 7.66 ± 1.26%. Control subjects did not significantly differ from patients with T1D in terms of age or BMI. Within the 34 patients, we selected 8 for the RNA-seq analysis, 50% female and 50% male, with a more stringent inclusion and exclusion criteria. Their age was 29.75 ± 5.85 years and their BMI was 22.50 ± 2.00 kg/m^2. They had been diagnosed with T1D at 27.13 ± 7.43 years, had a duration of the disease of 2.50 ± 1.98 years and a hemoglobin A1c level of 7.36 ± 2.07%. Specific information on each subject can be found in **Table 2**.

TABLE 1 | Data from the control subjects and patients with T1D recruited for the study.

	Control Subjects	Patients with T1D	*p* Value
N	24	34	—
Gender	11/24 (45.8%) Female 13/24 (54.2%) Male	17/34 (50%) Female 17/34 (50%) Male	—
Age (years)	30.46 ± 8.18	32.54 ± 8.96	0.4301
BMI (kg/m^2)	23.90 ± 2.87	23.80 ± 3.11	0.9075
Age at T1D diagnosis (years)	NA	20.79 ± 9.90	—
Duration of T1D (years)	NA	11.75 ± 9.70	—
HbA1c (%)	NA	7.66 ± 1.26	—

Data presented as mean ± SD; p value calculated from Mann–Whitney test.
T1D: type 1 diabetes; BMI, body mass index; NA, not applicable.

DC Differentiation Efficiency Is Similar in Patients with T1D and Control Subjects

Monocytes were isolated magnetically from PBMCs. The yield of monocyte isolation—calculated as the percentage of the absolute number of CD14$^+$ cells in the positively isolated fraction related to the absolute number of CD14$^+$ cells in PBMCs—was 54.95 ± 24.97% (mean ± SD) for control subjects and 56.62 ± 18.12% for patients with T1D. The percentage of purity of CD14$^+$ cells in the isolated fraction was 80.59 ± 10.18% for control subjects and 79.33 ± 7.56% for patients, and viability was 95.06 ± 4.14 and 94.92 ± 3.60%, respectively. The efficiency of differentiation to DCs at day 6 was 87.96 ± 6.61% for control subjects and 86.92 ± 6.86% for patients. No statistically significant differences were found when comparing these parameters between both groups. Data are detailed in **Table 3**.

PS-Liposomes Show Multivesicular Vesicle Morphology and Encapsulate Insulin Peptides

Liposomes were characterized in terms of diameter, polydispersity index (PdI), surface charge (ζ-potential) and efficiency of peptide encapsulation (**Table 4**). All liposomes had a final lipid concentration of 30 mM. All liposomes were large to guarantee efficient phagocytosis, displaying a diameter superior to 690 nm. The presence of PS molecules in liposomes was confirmed by the negative charge measured at the liposome surface by ζ-potential (−38 mV). Regarding specific features of PSA-liposomes ($n = 3$), the mean diameter was 690 ± 29 nm (mean ± SD), the PdI was 0.40 ± 0.28 and the ζ-potential was −38.57 ± 6.76 mV. The mean of peptide A (human insulin A chain) encapsulation efficiency was 39.74 ± 22.10%. As for PSB-liposomes ($n = 3$), they had a mean diameter of 788 ± 264 nm, the PdI was 0.52 ± 0.42 and the ζ-potential was −37.50 ± 7.16 mV, and the mean of peptide B (human insulin B chain) encapsulation efficiency was 93.19 ± 0.92%. Differences in peptide encapsulation efficiency (PSA *vs.* PSB) are due to amino acid composition and different solubility of insulin chains A (21

TABLE 2 | Data from the patients with T1D included in the RNA-seq experiment.

Patient number	Gender	Age (years)	BMI (kg/m^2)	Age at T1D diagnosis (years)	Duration of T1D (years)	HbA1c (%)
1	Male	23	21.2	19	4	6.7
2	Female	28	24.4	23	5	6.5
3	Male	28	23.0	25	3	7.4
4	Female	33	21.4	33	0.5	5.9
5	Female	35	24.4	34	1	6.4
6	Female	32	18.6	31	0.5	5.9
7	Male	38	24.2	36	1	12.2
8	Male	21	23.0	16	5	7.9
Mean ± SD		29.75 ± 5.85	22.50 ± 2.00	27.13 ± 7.43	2.50 ± 1.98	7.36 ± 2.07

Data presented as mean ± SD.
T1D, type 1 diabetes; BMI, body mass index.

aa) and B (30 aa) in phosphate buffered saline media. B chain is more positively charged than A chain at neutral pH, resulting in a higher encapsulation efficiency in negatively charged liposomes. PSA-liposomes and PSB-liposomes presented multivesicular vesicle morphology when cryo-TEM analysis was performed (**Figure 1**).

Fluorescent-labeled PS-liposomes ($n = 4$) showed a diameter of 836 ± 217 nm, a PdI of 0.32 ± 0.06 and a ζ-potential of −38.90 ± 2.52 mV. Their PS-free counterparts, fluorescent PC-liposomes ($n = 4$), had a diameter of 1665 ± 488 nm, a PdI of 0.32 ± 0.09 and a ζ-potential of −7.60 ± 2.68 mV (**Table 4**).

TABLE 3 | Data from monocyte's isolation and dendritic cell differentiation.

	Yield (day 1, %)	Purity (day 1, %)	Viability (day 1, %)	Differentiation efficiency (day 6, %)
Control subjects	54.95 ± 24.97	80.59 ± 10.18	95.06 ± 4.14	87.96 ± 6.61
Patients	56.62 ± 18.12	79.33 ± 7.56	94.92 ± 3.60	86.92 ± 6.86
p Value	0.3789	0.2286	0.3664	0.5766

Yield: % of the absolute number of CD14⁺ cells in the positively isolated fraction related to the absolute number of CD14⁺ cells in PBMCs. Purity: % of CD14⁺ cells in the isolated fraction. Viability: % of Annexin V⁻7aad⁻ cells. Differentiation efficiency: % of CD11c⁺ cells. Data presented as mean ± SD; p value calculated from Mann–Whitney test.
T1D, type 1 diabetes.

Human DCs Display Optimal Kinetics of PS-Liposomes Phagocytosis without Affecting Viability

A time course analysis was performed to determine PS-liposomes uptake kinetics (**Figure 2A**, upper left panel). The capture of empty fluorescent PS-liposomes by DCs was significantly higher at 37°C when compared to 4°C ($p < 0.001$), coming from either control subjects ($n = 5$) or patients ($n = 10$). This result is immunologically crucial and demonstrates that DCs engulf liposomes by an active mechanism of phagocytosis. PS-liposomes uptake kinetics were identical between control subjects and patients with T1D.

To indirectly assess the role of PS in phagocytosis, the same analysis was performed replacing PS-liposomes with PC-liposomes (**Figure 2A**, lower left panel). The percentages of empty PC-liposomes phagocytosis by DCs from control subjects ($n = 6$) and patients ($n = 9$) were significantly higher at 37°C when compared to 4°C starting at 2 h ($p < 0.0001$). The kinetics of the capture did not differ between control subjects and patients. When comparing uptake kinetics of PS- and PC-liposomes (Figure S1 in Supplementary Material), statistically significant differences were found, as expected. The presence of PS significantly accelerated phagocytosis in the first 2 h of co-culture ($p < 0.05$) both in control subjects and patients. In preliminary experiments, each type of liposome was tested in several sizes (diameter range 505–2,138 nm), and similar kinetics of capture were observed,

TABLE 4 | Features of the liposomes used in the study.

Liposome type	Diameter (nm)	Polydispersity index	ζ-potential (mV)	Encapsulation efficiency (%)
PSA-liposomes	690 ± 29	0.40 ± 0.28	−38.57 ± 6.76	39.74 ± 22.10
PSB-liposomes	788 ± 264	0.52 ± 0.42	−37.50 ± 7.16	93.19 ± 0.92
Fluorescent PS-liposomes	836 ± 217	0.32 ± 0.06	−38.90 ± 2.52	(empty)
Fluorescent PC-liposomes	1665 ± 488	0.32 ± 0.09	−7.60 ± 2.68	(empty)

Data presented as mean ± SD.

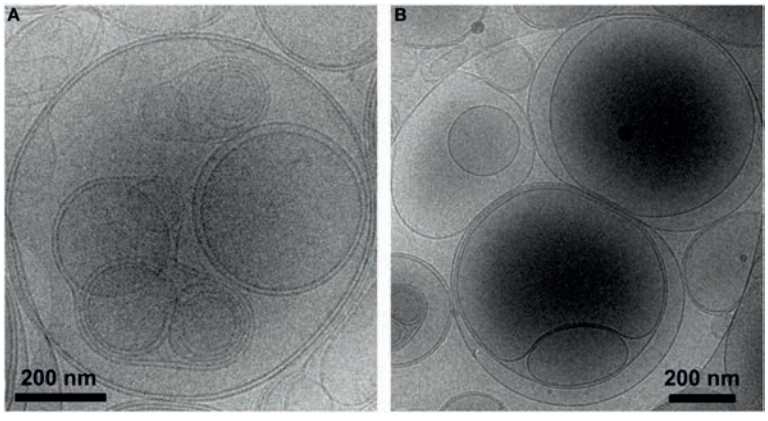

FIGURE 1 | PS-liposomes display multivesicular and multilamellar morphology. Cryogenic transmission electron microscopy images of **(A)** PSA-liposomes (left) and **(B)** PSB-liposomes (right). Bar = 0.2 μm.

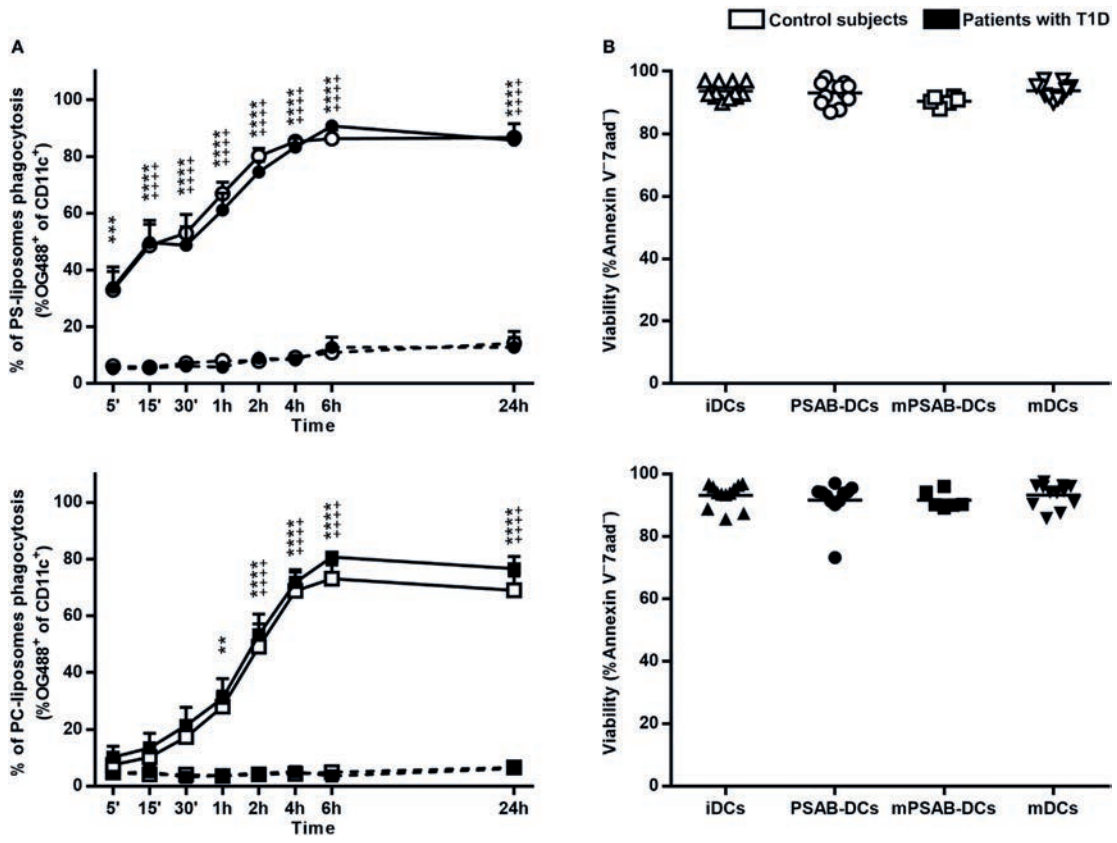

FIGURE 2 | Liposomes are efficiently phagocyted by dendritic cells (DCs) and preserve a high viability. **(A)** Uptake of liposomes fluorescently labeled with lipid-conjugated fluorescent dye Oregon Green 488 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine by DCs. Upper panel: time course of the capture of fluorescently-labeled PS-liposomes by DCs obtained from control subjects (white circles, $n = 5$) and patients with type 1 diabetes (T1D) (black circles, $n = 10$) at 37°C (continuous line) and at 4°C (discontinuous line). Results are mean ± SEM. Comparisons between phagocytosis by control subjects DCs at 37 and 4°C showed significant differences [$^{++++}p < 0.0001$, two-way analysis of variance (ANOVA)]; also, significant differences were found when comparing phagocytosis in patients with T1D at 37 and 4°C ($^{***}p < 0.001$, $^{****}p < 0.0001$, Two-way ANOVA). No differences were found when comparing PS-liposomes uptake by DCs from control subjects and patients with T1D (Two-way ANOVA). Lower panel: time course of the capture of fluorescently-labeled PC-liposomes by DCs obtained from control subjects (white squares, $n = 6$) and patients with T1D (black squares, $n = 9$) at 37°C (continuous line) and at 4°C (discontinuous line). Results are mean ± SEM. Comparisons between phagocytosis by control subjects DCs at 37 and 4°C showed significant differences ($^{++++}p < 0.0001$, Two-way ANOVA); also, significant differences were found when comparing phagocytosis in patients with T1D at 37 and 4°C ($^{**}p < 0.01$, $^{****}p < 0.0001$, Two-way ANOVA). No differences were found when comparing PC-liposomes uptake by DCs from control subjects and patients with T1D (Two-way ANOVA). **(B)** Viability of DCs from control subjects (upper panel, white symbols, $n \geq 6$) and patients with T1D (lower panel, black symbols, $n \geq 6$) assessed by annexin V and 7aad staining. Triangles represent immature DCs (iDCs), circles represent iDCs cultured with PSA-liposomes and PSB-liposomes (PSAB-DCs), squares represent mature PSAB-DCs (mPSAB-DCs) and upside-down triangles represent mature DCs (mDCs). MDCs were induced by culture with cytokine cocktail.

independently of liposome size (Figure S2 in Supplementary Material), thus confirming that PS is the key factor in accelerating phagocytosis.

The viability of the different conditions of DCs (iDCs, PSAB-DCs, mPSAB-DCs, and mDCs) was assessed to determine liposome toxicity. The mean viability for each condition was always >90%, both in DCs obtained from control subjects ($n \geq 6$) (**Figure 2B**, upper right panel), and patients with T1D ($n \geq 6$) (**Figure 2B**, lower right panel).

PS-Liposomes Uptake Regulates the Phenotypic Maturation of Human DCs

Changes in DCs phenotype were determined in control subjects ($n \geq 5$) and patients with T1D ($n \geq 8$). The membrane molecules assessed were: PS-receptors (CD36, TIM4, and αvβ5 integrin), antigen-presentation molecules (HLA-ABC and HLA-DR), adhesion molecules (CD54), costimulation molecules (CD40 and CD86), activation molecules (CD25), chemokine receptors (CCR7, CCR2, and CXCR4), and pattern recognition receptors

(TLR2, CD14, and DC-SIGN). **Figure 3** shows the relative Median of Fluorescence Intensity referred to mDCs.

PS-receptors CD36, TIM4 and αvβ5 integrin were expressed in iDCs. After liposome uptake, PSAB-DCs from patients decreased CD36 expression ($p < 0.05$) and upregulated TIM4 expression ($p < 0.05$) in comparison to iDCs, but PSAB-DCs presented a higher expression of CD36 and TIM4 than mDCs from control subjects and patients ($p < 0.05$). Moreover, PSAB-DCs from patients had increased levels of TIM4 in comparison to PSAB-DCs from control subjects ($p < 0.05$). The expression of αvβ5 integrin was higher in PSAB-DCs than in mDCs in patients ($p < 0.05$). As expected, CD36 and TIM4 were downmodulated in mDCs ($p < 0.05$), and αvβ5 integrin showed the same tendency.

Regarding HLA molecules, HLA-ABC was expressed similarly in iDCs and mDCs from both groups, and decreased in PSAB-DCs from patients after liposome capture ($p < 0.05$) —and control subjects displayed the same tendency. Concerning HLA-DR, iDCs showed a lower expression of this marker when compared to mDCs ($p < 0.001$). After liposome phagocytosis (PSAB-DCs), the low HLA-DR levels were preserved. As for the expression of adhesion molecule CD54, it was lower in iDCs from patients in comparison to mDCs ($p < 0.05$), and control subjects displayed the same tendency. After liposome uptake, no changes in CD54 expression were observed in PSAB-DCs when compared to iDCs, but mDCs displayed increased levels of CD54 in comparison to PSAB-DCs ($p < 0.05$). The expression of CD54 was higher in mPSAB-DCs exposed to a maturation stimulus despite the uptake of liposomes ($p < 0.05$).

Expression of costimulatory molecules CD40 and CD86 was lower in iDCs than in mDCs ($p < 0.01$). Liposome phagocytosis did not increase the expression of these molecules in PSAB-DCs. Moreover, PSAB-DCs presented lower levels of these markers when compared to mDCs ($p < 0.0001$), and even when exposed to pro-inflammatory stimulus (mPSAB-DCs) in comparison to mDCs ($p < 0.05$). Regarding the expression of activation marker CD25, it was lower in iDCs when compared to mDCs ($p < 0.0001$). Upregulation of CD25 was observed after liposome uptake in PSAB-DCs from control subjects ($p < 0.01$), but remained unaltered in patients. PSAB-DCs from both groups presented CD25 downmodulated when compared to mDCs ($p < 0.001$). Furthermore, DCs loaded with liposomes and exposed to pro-inflammatory stimulus (mPSAB-DCs) displayed lower levels of CD25 than mDCs in patients with T1D ($p < 0.01$).

Chemokine receptors CCR7 and CCR2 were expressed in iDCs. After liposome capture, the expression of both molecules increased in patients with T1D ($p < 0.05$). CCR7 was upregulated in PSAB-DCs when compared to mDCs in patients ($p < 0.05$), and control subjects displayed the same tendency. CXCR4, overexpressed in mDCs in comparison to iDCs ($p < 0.05$), was maintained low after liposome engulfment (PSAB-DCs). The expression of CXCR4 was higher in DCs exposed to a maturation stimulus despite the uptake of liposomes (mPSAB-DCs) ($p < 0.05$).

Pattern recognition receptors were assessed in DCs. TLR2 expression was similar in all experimental conditions, despite showing a tendency to increase after liposome phagocytosis (PSAB-DCs) in patients. CD14 was similarly expressed in iDCs and mDCs, but liposome uptake and maturation stimulus induced downregulation of this marker ($p < 0.05$). DC-SIGN, expressed in iDCs, displayed a tendency to be downmodulated after liposome capture (PSAB-DCs), especially in controls, which was more marked after a pro-inflammatory stimulus. Nonetheless, this marker showed a tendency to remain higher in PSAB-DCs than in mDCs in patients.

In terms of cytokine secretion by DCs from patients ($n \geq 3$) and control subjects ($n \geq 3$) (**Figure 4**), IL-6 was released in low amounts after liposome phagocytosis and, as expected, its secretion increased after maturation stimulus. TNF-α was not increased after liposome uptake and its secretion increased in pro-inflammatory conditions. Liposome engulfment maintained a high profile of TGF-β1 secretion both in control subjects and patients, and tended to decrease in mPSAB-DCs and mDCs, although non-significant. Regarding IL-10 production, PSAB-DCs displayed a tendency to increase the secretion, although non-statistically significant, in patients with T1D. IL-2, IL-17A, and IFN-γ were not detected in any condition of the assay (data below the detection limit). IL-4 was not considered as it was used in culture media for DC differentiation.

PS-Liposomes Uptake Impairs DCs Ability to Stimulate Autologous T Cell Proliferation

DCs derived from patients with T1D ($n \geq 12$) and control subjects ($n = 12$) induced similar levels of autologous T cell proliferation (**Figure 5**). As expected, $CD4^+$ T cell proliferation induced by mDCs was higher than proliferation induced by iDCs in both groups ($p < 0.01$). $CD8^+$ T cell proliferation induced by mDCs was higher than proliferation induced by iDCs in control subjects ($p < 0.05$), but not in patients. Importantly, the capture of PSAB-liposomes by iDCs did not increase autologous $CD4^+$ and $CD8^+$ T cell proliferation, both in patients and control subjects. Moreover, a significant decrease of $CD8^+$ T cell proliferation induced by PSAB-DCs from patients was observed after liposome capture, when compared to iDCs ($p < 0.01$). This effect was reverted after DCs maturation.

In terms of cytokine production, PBMCs co-cultured with PSAB-DCs displayed a cytokine profile (IL-6 and IFN-γ) similar to iDCs (**Figure 5**). Interestingly, PBMCs showed a tendency to increase IFN-γ and IL-6 secretion when co-cultured with mPSAB-DCs or mDCs, respectively, only in patients with T1D. IL-2, IL-4, IL-10, IL-17A, and TNF-α were not detected in any condition of the assay (data below the detection limit).

Transcriptional Changes in DCs from Patients with T1D after PS-Liposomes Phagocytosis Point to an Immunoregulatory Prolife

RNA-seq analysis was performed in DCs from 8 patients with T1D (**Table 2**) in order to identify transcriptional changes after the capture of PS-liposomes. Phagocytosis was verified by flow cytometry using fluorescent liposomes. After 4 h of co-culture, 73.88 ± 11.57% (mean ± SD) of DCs were positive for fluorescent signal.

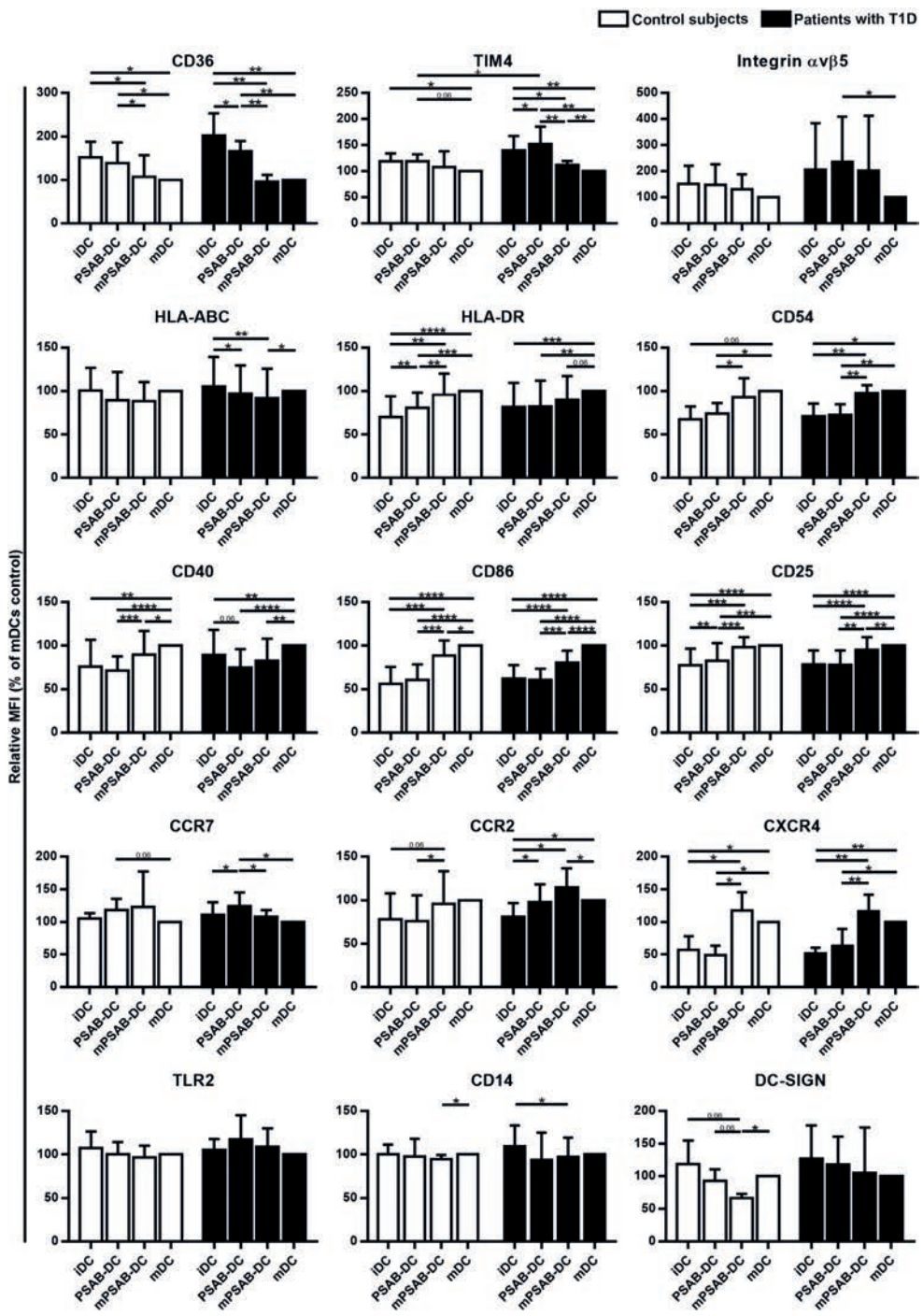

FIGURE 3 | Continued

FIGURE 3 | Capture of PSA-liposomes and PSB-liposomes regulates dendritic cell (DCs) phenotype. Relative CD36, TIM4, Integrin αvβ5, HLA-ABC, HLA-DR, CD54, CD40, CD86, CD25, CCR7, CCR2, CXCR4, TLR2, CD14, and DC-SIGN membrane expression in DCs obtained from control subjects (white bars, $n \geq 5$) and patients with type 1 diabetes (T1D) (black bars, $n \geq 8$). Bars represent immature DCs (iDCs), iDCs after the capture of PSA-liposomes and PSB-liposomes (PSAB-DCs), mature PSAB-DCs (mPSAB-DCs), or mature DCs (mDCs), 24 h after culture. MDCs were induced by culture with cytokine cocktail. Data presented as mean ± SD of relative Median of Fluorescence Intensity (MFI), this being MFI of each culture condition referred to their respective mDCs control. Significant differences were found when comparing culture conditions in the same group of subjects (*$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, Wilcoxon test), and when comparing the same culture conditions between patients with T1D and control subjects ($^{+}p < 0.05$, Mann–Whitney test).

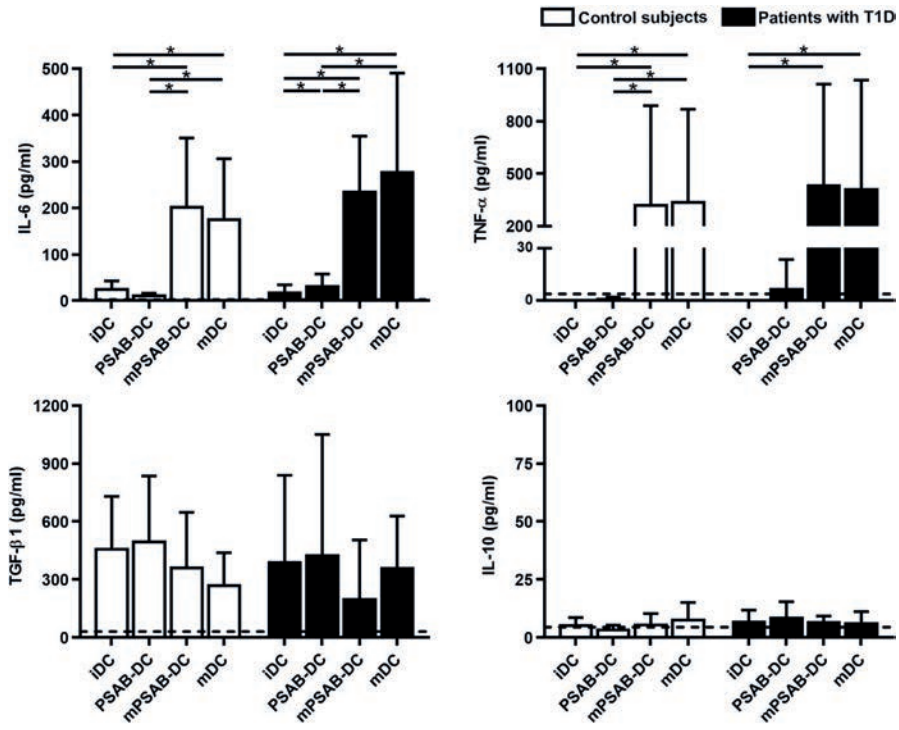

FIGURE 4 | The uptake of PSAB-liposomes by dendritic cells (DCs) does not alter cytokine profile. Concentration (pg/ml) of IL-6, TNF-α, TGF-β1, and IL-10 secreted by DCs obtained from control subjects (white bars, $n \geq 3$) and patients with type 1 diabetes (T1D) (black bars, $n \geq 3$). Bars represent immature DCs (iDCs), iDCs after the capture of PSA-liposomes and PSB-liposomes (PSAB-DCs), mature PSAB-DCs (mPSAB-DCs), or mature DCs (mDCs). MDCs were induced by culture with cytokine cocktail. Data presented as mean ± SD. Significant differences were found when comparing the different conditions in the same group of subjects (*$p < 0.05$, Wilcoxon test), and differences were not found when comparing the same culture conditions between patients with T1D and control subjects (Mann–Whitney test).

Integrity of the isolated RNA material was assessed for each sample, being optimal for RNA-seq experiment: RIN 9.0 ± 0.56 (mean ± SD). Bioinformatics analysis of the RNA-seq experiment revealed that only 233 of 22,711 genes detected were differentially expressed between iDCs and PSAB-DCs (p value < 0.0013, adjusted p value < 0.1254). Of these 233 genes, 203 (87.12%) were downregulated and the remaining 30 (12.88%) were upregulated, and 224 corresponded to protein-coding genes. Despite the heterogeneous basal transcriptomics of DCs from eight patients, gene expression was clearly altered toward a similar profile after PS-liposomes phagocytosis (Figure S3 in Supplementary Material).

We analyzed several categories and molecules related to DC function (Table S1 in Supplementary Material). DEGs were mainly related to metabolism, gene expression, immunoregulation, signal transduction, molecule transport, post-translational protein modification, cytokine signaling, cell cycle, vesicle-mediated processes, DNA replication and repair, antigen processing and presentation, apoptosis, and cytoskeleton organization (**Table 5**). Due to the immunotherapeutic potential of PS-liposomes, DEGs involved in tolerance were analyzed in detail. DEGs linked to the immune system were primarily downregulated and involved in antigen processing and presentation (*KBTBD6*, *BTK*, *CDC23*, *UBE2E3*, *CD1D*), regulation of the immune response (*DAPP1*,

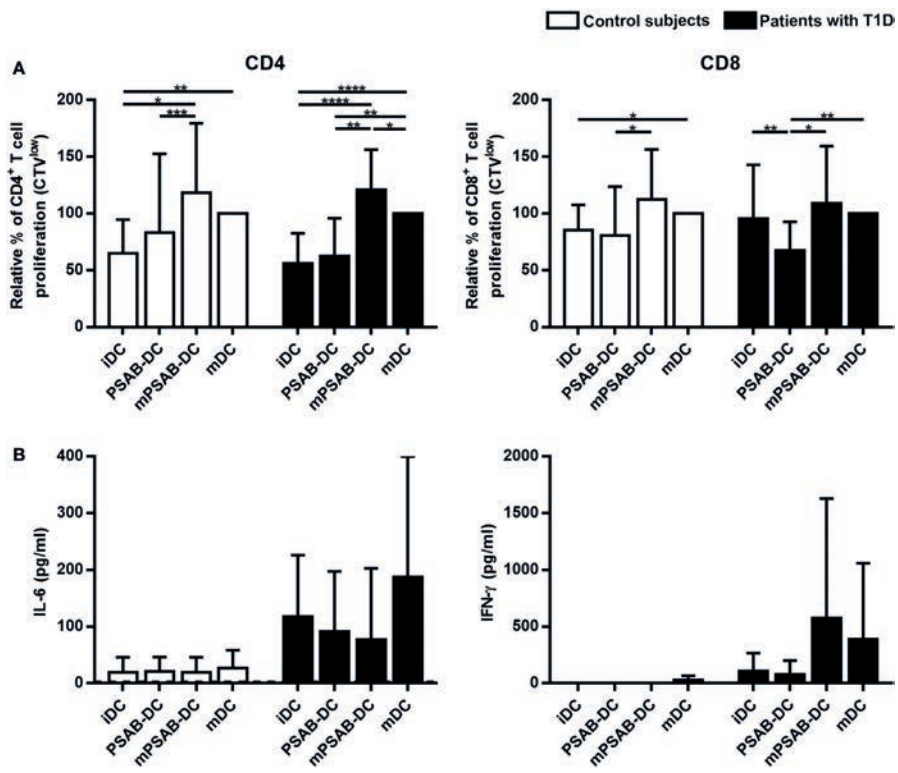

FIGURE 5 | Capture of PSA-liposomes and PSB-liposomes affects dendritic cells (DCs) functionality. **(A)** Relative autologous proliferation of $CD3^+CD4^+$ and $CD3^+CD8^+$ subsets induced by DCs obtained from control subjects (white bars, $n = 12$) and patients with type 1 diabetes (T1D) (black bars, $n \geq 12$). Autologous peripheral blood mononuclear cells were stained with CellTrace Violet (CTV) and co-cultured at 1:10 ratio for 6 days with each condition of DCs, and proliferation was measured as the percentage of CTV^{low} cells. Bars represent immature DCs (iDCs), iDCs after the capture of PSA-liposomes and PSB-liposomes (PSAB-DCs), mature PSAB-DCs (mPSAB-DC), or mature DCs (mDCs). MDCs were induced by culture with cytokine cocktail. Data presented as mean ± SD of relative proliferation induction, this being the percentage of CTV^{low} cells in each co-culture condition referred to that of their respective mDCs control. Significant differences were found when comparing culture conditions in the same group of subjects ($^*p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Wilcoxon test), and differences were not found when comparing the same culture conditions between patients with T1D and control subjects (Mann-Whitney test). **(B)** IL-6 and IFN-γ secretion (pg/ml) assessed in supernatants of autologous proliferation co-culture with cells from control subjects (white bars, $n \geq 3$) and patients with T1D (black bars, $n \geq 3$). Bars represent immature DCs (iDCs), iDCs after the capture of PSA-liposomes and PSB-liposomes (PSAB-DCs), mature PSAB-DCs (mPSAB-DC), or mature DCs (mDCs). MDCs were induced by culture with cytokine cocktail. Data presented as mean ± SD. No differences were found when comparing the different conditions in the same group of subjects (Wilcoxon test), or when comparing the same culture conditions between patients with T1D and control subjects (Mann–Whitney test).

GIMAP4, *SLAMF6*) and cytokine signaling relevant in the interaction between T cells and DCs (*SOCS2*, *TNFRSF11A*). However, although very few genes were upregulated after PS-liposomes phagocytosis, these were related to the prevention of DC maturation [*TNFSF14*, *TNFAIP3*, *VEGFA*, *SHB*, leukocyte associated immunoglobulin-like receptor 1 (*LAIR1*), *NFKBIA*]. Also, genes related to apoptosis were downregulated in DCs after liposome uptake (*BLCAP*, *PMP22*, *LMNB1*).

Validation by qRT-PCR of the selected gene targets (*CYTH4*, *GIMAP4*, *HPGD*, *NFKBIA*, *PLAUR*, *TNFAIP3*, *TNFSF14*, and *VEGFA*) confirmed the RNA-seq results, when tested in DCs from 10 patients with T1D (**Figure 6**). As expected, gene expression analysis in DCs from 9 control subjects showed the same pattern. Regarding mDCs—from 4 patients with T1D and 3 control subjects—we observed a seemingly different gene expression pattern when compared to PSAB-DCs — and with iDCs. Genes upregulated by PS-liposomes, such as *CYTH4* and *TNFSF14*, tended to be downregulated in mDCs; other genes tended to be differentially expressed in mDCs (*NFKBIA*, *PLAUR*, *TNFAIP3*, *GIMAP4*, *VEGFA*, and *HPGD*) in comparison to the other conditions.

DISCUSSION

Apoptosis is a key factor in the maintenance of immunological tolerance. The uptake of apoptotic cells, through a process called efferocytosis, results in tolerogenic presentation of autoantigens inducing specific tolerance rather than autoimmunity (14).

TABLE 5 | DEGs in dendritic cells (DCs) from patients with type 1 diabetes (T1D) after PSA-liposomes and PSB-liposomes phagocytosis.

Category	Number of DEGs	*P* value	Representative downregulated genes	Representative upregulated genes
Adhesion	3	≤0.001076	*SCYL3, MEGF9*	*IGSF9*
Antigen processing and presentation	7	≤0.001024	*KBTBD6, BTK, CDC23, UBE2E3, CD1D, CUL3, KIF11*	
Apoptosis	6	≤0.000593	*BLCAP, PMP22, LMNB1, CASP3, DCAF7, BCL2L1*	
Cell cycle	9	≤0.001162	*CSRP2BP, BUB1, MCPH1, CDK13, PCNA, MCM4, SMC2, NCAPG2, AURKA*	
Cytokine signaling	9	≤0.001191	*TRIM5, SOCS2, STX3, TNFRSF11A, NUP160*	*TNFSF14, VEGFA, TNF, IFNLR1*
Cytoskeleton organization	6	≤0.000897	*MAPRE2, RMDN1, CKAP2, MDM1, RCSD1, CDC42SE1*	
DNA replication and repair	8	≤0.000995	*WRNIP1, PAXIP1, MSH2, RAD51C, DCLRE1A, ALKBH1, PARG, MLH1*	
Gene expression	36	≤0.001293	*ZNF436, MYB, ZFP36L2, MIER3, ZBTB5, HHEX, GTF2B, DYRK2, NFIA, ZBTB39*	
Immunoregulation	25	≤0.001146	*GIMAP4, SLAMF6, DAPP1, MEF2C, BST1, PROS1, MNDA*	*TNFAIP3, PLAUR, NFKBIA*
Metabolism	43	≤0.001225	*C9orf64, HPGD, TIMMDC1, ICK, DDO, DCTD, CDYL2, GLRX, TPK1*	*MFSD2A*
Molecule transport	14	≤0.001252	*ERLIN1, SLC10A7, UNC50, ATP10D, SLC40A1, CLCN3, STIM2*	*SLC43A3, SLCO4A1, CLCN6*
Post-translational protein modification	13	≤0.001056	*FBXO36, NSMCE4A, VWA5A, FBXO25, DCAF12, CBX4, RMND5A, LNX2, BTBD3*	*PPME1*
Signal transduction	18	≤0.001298	*SNN, SKI, PAQR8, UBFD1, N4BP1, FZD5, NET1, ZBED3, FRAT2*	*SHB*
Vesicle-mediated processes	8	≤0.001208	*GOLPH3L, SEC22C, RAB32, EHBP1, KIF20B, SNX18*	*CYTH4, LDLR*

DEGs, differentially expressed genes.

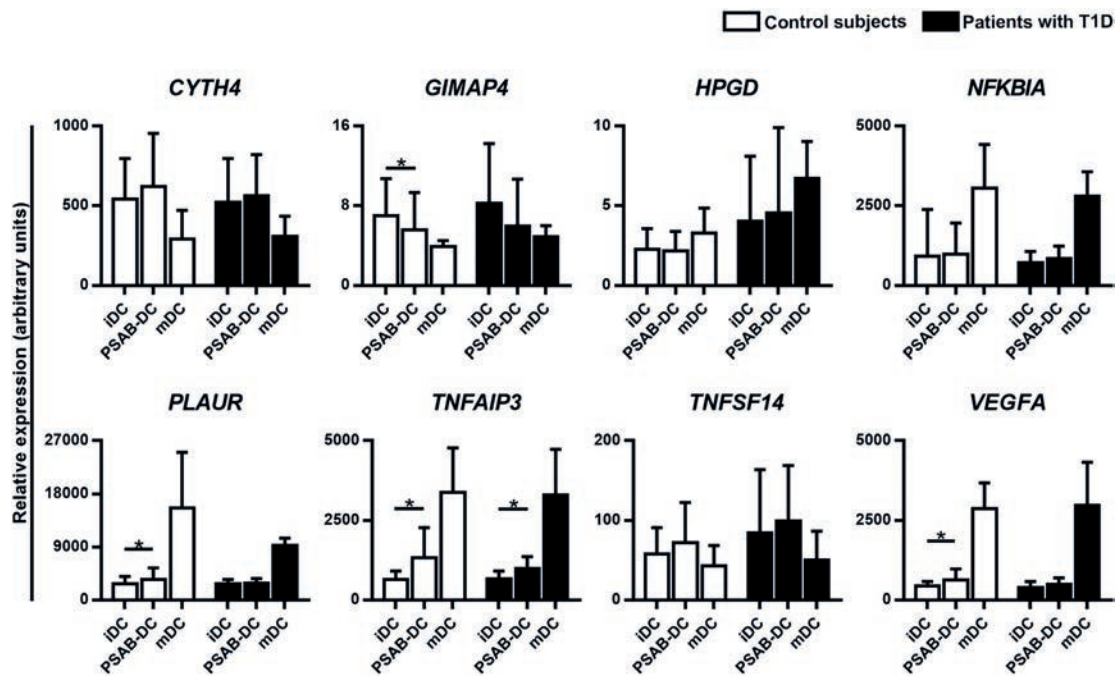

FIGURE 6 | Quantitative RT-PCR validates the RNA-seq results. Relative gene expression of 8 selected targets in immature dendritic cells (iDCs), after phagocytosis of PSA-liposomes and PSB-liposomes (PSAB-DCs), and in mature dendritic cells (mDCs), analyzed by quantitative RT-PCR. Gene expression signals were normalized to *GAPDH*. Bars show the mean ± SD of gene expression in control subjects (white bars, $n \geq 3$) and patients with type 1 diabetes (T1D) (black bars, $n \geq 4$). Statistically significant differences were found when comparing the different conditions in the same group of subjects (*$p < 0.05$, Wilcoxon test), and differences were not found when comparing the same culture conditions between patients with T1D and control subjects (Mann–Whitney test).

Therefore, the inherent immunomodulatory properties of apoptotic cells can be useful to design innovative immunotherapies. Based on this tolerogenic potential of efferocytosis, we generated liposomes that mimic apoptotic β-cells. At present, liposomes are clinically used mainly as vehicles for drugs (29–31), but they can be designed to modulate immune responses. This liposome-based immunotherapy resembles apoptotic cells and acts through the immunosuppressive signal of PS (32) and tolerogenic autoantigen presentation. These large PS-liposomes, after phagocytosis, are effective in restoring self-tolerance in experimental autoimmune diseases (16, 17) by their interaction with DCs and the arrest of the autoimmune reaction. To explore the clinical potential of this strategy, we have determined the effect of PS-liposomes loaded with human insulin peptides in DCs from patients with T1D. This effect has been assessed in several aspects: phagocytosis, phenotypic changes, effect on T cell proliferation and cytokine profile.

Regarding phagocytosis, lipid membrane composition is crucial for rapid engulfment by DCs, as demonstrated using PS- and PC-liposomes. As expected, the presence of PS accelerated phagocytosis of liposomes both in control subjects and patients with a dynamic typical of apoptotic cell clearance. PS-liposomes were more efficiently engulfed by DCs than the equivalent ones without PS in the first 2 h of co-culture, reaching plateau after 6 h. When encountering PS-liposomes, DCs are deceived into sensing that they are actual apoptotic cells that need to be rapidly efferocyted in order to avoid secondary necrosis that could contribute to autoimmunity (33, 34). Moreover, the preservation of DCs viability proved that PS-liposomes are not toxic, as reported for other types of liposomes (29–32).

The second aspect was the assessment of DCs phenotype. After liposome engulfment, PSAB-DCs maintained high levels of PS-receptors that mediate this uptake, when compared to mDCs, pointing to the preservation of phagocytosis ability in tolerogenic DCs (tolDCs). Interestingly, the upregulation of TIM4 expression observed in PSAB-DCs from patients might contribute to a positive feedback of phagocytosis. Upon maturation, PS-receptors were downmodulated correlating with the phagocytic capacity of mDCs, as described (35). The expression pattern of molecules involved in antigen presentation (HLA, costimulatory and adhesion molecules) in PSAB-DCs concurs with a tolerogenic function, both in patients and control subjects. The expression of CD25 activation marker, linked to DCs activation and autoimmunity (36, 37), confirmed the intermediate activation status of PSAB-DCs after phagocytosis. Also, the chemokine receptors expression pattern supports DCs ability to drive their migration to secondary lymphoid tissues (38, 39), and moreover, the high CCR7 expression is associated with induction of tolerance after efferocytosis (40). Additionally, the expression of pattern recognition receptors was not altered by liposomes, as described for human DCs (32). This phenotype is similar to the previously observed in mice (16, 17). Of note, RNA-seq analysis reinforces these results. Furthermore, upon liposome capture, the immunomodulatory cytokine TGFβ-1 was secreted, a reported effect driven by PS (34) that could suppress DC maturation and define the T cell response afterward. As expected, liposome capture did not induce IL-6 nor TNF-α secretion by DCs, but maturation did. Overall, the results point to the tolerogenic effect of these vesicles, which act on re-establishing self-tolerance. We observed minor phenotypic differences between DCs from patients and control subjects, which could be due to epigenetic changes caused by autoimmunity and metabolic dysregulation (41–43).

The third aspect was the analysis of autologous T cell proliferation induced by PSAB-DCs. In agreement with DCs phenotype, T cell proliferation induced by PSAB-DCs was similar or even lower than the induced by iDCs, both in patients and control subjects. Interestingly, in patients with T1D, there was a significant reduction in $CD8^+$ T cell subset proliferation induced by tolDCs when compared to iDCs. This effect could be related to a reduction of the T cell cytotoxic activity, the most important effector response in human T1D (44, 45). In fact, after efferocytosis, DCs present apoptotic cell autoantigens to cognate T cells in the absence of costimulation, favoring tolerance to self (12, 13). It is reasonable to think that liposomes mimicking apoptotic cells will cause a similar effect, Additional studies using tetramers would be relevant to determine the antigen-specificity of the T cells involved in tolerance induction, even in pro-inflammatory conditions, in which T cells seem to proliferate more vigorously. Cytokines produced during the autologous T cell proliferation assay induced by PSAB-DCs discard a Th1 and Th17 profile, which could be detrimental in the induction of tolerance. In fact, IFN-γ, which is involved in a Th1 response, and IL-6 secretion, which partially contributes to induce Th17 response in T1D (46), remain poorly secreted in co-cultures of PBMCs with PSAB-DCs. Interestingly, higher amounts of IL-6 and IFN-γ tend to be produced by mDCs from patients with T1D when compared to controls. This feature could reflect the ongoing autoimmune reaction, present in peripheral blood from patients (47).

One of the obstacles of tolerogenic therapies in human disease is the heterogeneity of the *ex vivo*-generated tolDCs, which vary depending on the source, the manufacturing protocols, and the timespan of the experiment. Work is in progress to define and standardize a set of phenotypical and functional characteristics of tolDCs (48). To date, tolDCs are accepted as maturation-reluctant cells with low expression of antigen-presenting and costimulatory molecules and a tolerogenic-skewed cytokine profile (49). In this sense, one of the advantages of direct administration of the liposomes reported herein would be the generation of tolDCs *in vivo*, avoiding *ex vivo* cell manipulation. Our previous results in mice demonstrate this hypothesis (16, 17). However, a global picture of changes induced by PS-liposomes phagocytosis would grant a better understanding of tolerogenicity.

To fully characterize the immunomodulatory effects of liposomes, transcriptomic analysis was performed in DCs. Eight patients with a short T1D duration were selected in order to minimize the influence of long-term hyperglycemia on immune response, as reported (41–43). RNA-seq revealed a set of DEGs that avoid DCs maturation and contribute to tolerogenic antigen presentation. One of the most hyperexpressed genes was the vascular endothelial growth factor (VEGF) A (*VEGFA*), involved in cytokine signaling after efferocytosis (50), iDCs recruitment and maturation inhibition (51). VEGF increases the expression of the *TNFSF14* gene (52), also upregulated by PS-liposomes (53). In turn, TNFSF14 upregulates the production of TGF-β by phagocytes (54), and upon interacting with its ligand in T cells,

TNFSF14 regulates T cell proliferation (55), inducing local immunosuppression (56). Supporting this fact, apoptotic cell clearance has been described to inhibit inflammation *via* TGF-β and VEGF production (34). Additionally, VEGFA enhances the expression of indoleamine 2,3-dioxygenase (*IDO*) (57), which in turn codifies for an immunomodulatory enzyme expressed in tolDCs (58). Moreover, the hematopoietically expressed homeobox (*HHEX*) gene, a repressor of VEGF signaling (59), is downregulated after PS-liposomes phagocytosis, whereas an inductor of VEGF expression, activating transcription factor 4 (*ATF4*) (60), is upregulated. Furthermore, the hyperexpressed SH2 domain containing adaptor protein B (*SHB*) gene codifies for a protein that regulates VEGF-dependent cellular migration (61), Th2 polarization and T regulatory cell induction (62). Other upregulated tolerogenic genes, such as the TNF alpha induced protein 3 (*TNFAIP3*) and the *LAIR1*, can inhibit DC maturation and their deficiency causes autoimmune and autoinflammatory diseases (63–66). In the same way, the hyperexpression of the *NFKBIA* gene would contribute to inhibit DC maturation and T cell activation (67, 68). Regarding cytokine signature, our results agree with those found in phenotypic and functional experiments. A relevant cytokine for tolerance induction is TGF-β1, secreted after efferocytosis (69). After PS-liposomes capture, DCs showed a biological increase of TGF-β1 transcription, although non-significant, probably due to the short timespan of the experiment (70). In fact, TGF-β1 was found in culture supernatants 24 h after PS-liposomes phagocytosis. Also, the immunoregulatory interferon lambda receptor 1 (*IFNLR1*) gene is one of the few overexpressed in DCs after PS-liposomes uptake. This receptor induces tolDCs that promote regulatory T cell expansion (71). Unexpectedly, the *TNF*-encoding gene was upregulated in DCs after liposome phagocytosis, and the same tendency was observed in protein secretion. Nevertheless, this behavior was very different to that observed in mDCs, which secreted higher amounts of TNF. The increase of TNF gene expression in our RNA-seq agrees with the upregulation of *TNFSF14* and *TNFAIP3* genes. Furthermore, a critical role for TNF has been reported in human tolDCs in the induction of antigen-specific regulatory T cells (72). Also, our previous results showed that murine tolDCs upregulated TNF-gene expression after efferocytosis (14). Overall, these results are consistent with the pleiotropic effects of TNF. Furthermore, in our previous research, PGE_2 was found to be crucial in tolerance induced by PS-liposomes in mice (16). Strikingly, this pathway does not seem to be upregulated in human DCs, probably due to divergences between mice and men. Nevertheless, our data indirectly point to the involvement of the PGE_2 pathway in human DCs: first, the downregulation of the hydroxyprostaglandin dehydrogenase 15-(NAD) (*HPGD*) gene, involved in PGE_2 degradation, and second, a biological upregulation (although non-significant) of the peroxisome proliferator activated receptor gamma (PPARG), a gene induced by prostaglandins which is a negative regulator of pro-inflammatory cytokines (73). Furthermore, PGE_2 has been described to stimulate the synthesis of VEGF (74). In summary, comparative transcriptome studies identify the whole molecular features of tolDCs rather than describe a simple state of maturation or lack thereof in terms of phenotype and function (75). Further studies are required to find a common signature of tolerogenicity, a fact hindered by individual differences of human DCs and the heterogeneous results obtained with different agents used to promote tolDCs. Our findings describe the specific gene signature of PS-liposomes-induced tolDCs. Their genomic program, which drives a different functionality than those of iDCs and mDCs, contributes to dissect the complexity of tolerance regulation.

Perhaps not so peculiarly, most of the alterations found in DCs after PS-liposomes capture are also physiopathological strategies used by tumor cells to escape immune surveillance. Small vesicles rich in PS are released by tumor cells and act as immunosuppressive agents to inhibit tumor antigen-specific T cells (76). Tumor cells can induce immunological tolerance using mechanisms characteristic of apoptotic cell clearance, and PS-liposomes seem to make use of the same pathways to achieve similar effects.

The use of PS-liposomes filled with autoantigens is an innovative strategy to arrest autoimmunity by restoring tolerance to self. As a whole, our results support the tolerogenic behavior of DCs, induced by the phagocytosis of PS-liposomes, and suggest that, in the context of autoimmunity, they could act silencing potential autoreactive T cells. This process could possibly be an active silencer, and not only a lack of maturation of DCs. In summary, here we unveil a picture of efferocytosis mimicry that leads to phenotypic and functional changes in human DCs, accountable for tolerance induction. The herein reported results reinforce the potential of this biocompatible immunotherapy to re-establish immunological tolerance, opening the door to new therapeutic approaches in the field of antigen-specific autoimmune disorders.

ETHICS STATEMENT

This study was carried out after the approval and in strict accordance with the recommendations of the guidelines of Germans Trias i Pujol Ethical Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SR-F, IP-A, MC-S, FV, JV, and MV-P designed the study and interpreted the data; SR-F, DM, JV, and MV-P wrote the manuscript; SR-F, IP-A, DP-B, MC-S, SG-J, and AV performed the experiments; IP-A, SR-F, DM, and MV-P supervised the experiments; SR-F, EA, FV, and MV-P selected the patients and supervised the collection of blood samples; and SR-F, IP-A, FB, and AS analyzed the data. All authors revised the work and gave final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00253/full#supplementary-material.

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Conflict of Interest Statement: IP, MC, JV, DM, and MV are inventors in a patent (WO2015107140) that describes the use of autoantigen-encapsulating liposomes for the prevention or treatment of autoimmune disorders.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. DEGs in DCs from patients with T1D after phagocytosis of PSAB-liposomes, according to categories based on biological processes collected through different databases

Gene Symbol	Gene ID	Gene Name	Log_2 FC	*p*-value
ADHESION				
SCYL3	57147	SCY1 like pseudokinase 3	–0.08	0.000396
MEGF9	1955	Multiple EGF like domains 9	–0.52	0.000656
IGSF9	57549	Immunoglobulin superfamily member 9	0.18	0.001076
ANTIGEN PROCESSING AND PRESENTATION				
KBTBD6	89890	Kelch repeat and BTB domain containing 6	–0.15	0.000002
BTK	695	Bruton tyrosine kinase	–0.16	0.000291
CDC23	8697	Cell division cycle 23	–0.22	0.000397
UBE2E3	10477	Ubiquitin conjugating enzyme E2 E3	–0.19	0.000417
CD1D	912	CD1d molecule	–0.61	0.000595
CUL3	8452	Cullin 3	–0.22	0.000731
KIF11	3832	Kinesin family member 11	–0.12	0.001024
APOPTOSIS				
BLCAP	10904	Bladder cancer associated protein	–0.17	0.000175
PMP22	5376	Peripheral myelin protein 22	–0.33	0.000251
LMNB1	4001	Lamin B1	–0.26	0.000417
CASP3	836	Caspase 3	–0.37	0.000449
DCAF7	10238	DDB1 and CUL4 associated factor 7	–0.26	0.000488
BCL2L11	10018	BCL2 like 11	–0.41	0.000593
CELL CYCLE				
CSRP2BP	57325	Cysteine-rich protein 2-binding protein	–0.16	0.000111
BUB1	699	BUB1 mitotic checkpoint serine/threonine kinase	–0.06	0.000269
MCPH1	79648	Microcephalin 1	–0.10	0.000337
CDK13	8621	Cyclin dependent kinase 13	–0.15	0.000424

Gene Symbol	Gene ID	Gene Name	Log_2 FC	*p*-value
PCNA	5111	Proliferating cell nuclear antigen	–0.41	0.000691
MCM4	4173	Minichromosome maintenance complex component 4	–0.16	0.000925
SMC2	10592	Structural maintenance of chromosomes 2	–0.25	0.000946
NCAPG2	54892	Non-SMC condensin II complex subunit G2	–0.08	0.001090
AURKA	6790	Aurora kinase A	–0.26	0.001162
CYTOKINE SIGNALLING				
TNFSF14	8740	TNF superfamily member 14	0.61	0.000003
VEGFA	7422	Vascular endothelial growth factor A	0.30	0.000025
TRIM5	85363	Tripartite motif containing 5	–0.23	0.000047
SOCS2	8835	Suppressor of cytokine signalling 2	–0.12	0.000054
STX3	6809	Syntaxin 3	–0.28	0.000121
TNF	7124	Tumor necrosis factor	0.48	0.000175
IFNLR1	163702	Interferon lambda receptor 1	0.16	0.000193
TNFRSF11A	8792	Tumor necrosis factor receptor superfamily member 11A	–0.17	0.000677
NUP160	23279	Nucleoporin 160	–0.15	0.001191
CYTOSKELETON ORGANISATION				
MAPRE2	10982	Microtubule associated protein RP/EB family member 2	–0.16	0.000163
RMDN1	51115	Regulator of microtubule dynamics 1	–0.15	0.000238
CKAP2	26586	Cytoskeleton associated protein 2	–0.31	0.000320
MDM1	56890	Mdm1 nuclear protein	–0.13	0.000504
RCSD1	92241	RCSD domain containing 1	–0.34	0.000801
CDC42SE1	56882	CDC42 small effector 1	–0.21	0.000897
DNA REPLICATION AND REPAIR				
WRNIP1	56897	Werner helicase interacting protein 1	–0.12	0.000017
PAXIP1	22976	PAX interacting protein 1	–0.09	0.000028
MSH2	4436	DNA mismatch repair protein Msh2	–0.25	0.000188
RAD51C	5889	RAD51 paralog C	–0.09	0.000374
DCLRE1A	9937	DNA cross-link repair 1A	–0.15	0.000586
ALKBH1	8846	ALKB homolog 1, histone H2A dioxygenase	–0.11	0.000912
PARG	8505	Poly(ADP-ribose) glycohydrolase	–0.16	0.000985
MLH1	4292	MutL homolog 1	–0.16	0.000995
EXTRACELLULAR MATRIX ORGANISATION				
MFAP1	4236	Microfibril associated protein 1	–0.19	0.000130
SPARC	6678	Secreted protein acidic and cysteine rich	–0.24	0.001197

Gene Symbol	Gene ID	Gene Name	Log_2 FC	*p*-value
GENE EXPRESSION				
ZNF436	80818	Zinc finger protein 436	–0.16	0.000001
MYB	4602	MYB proto-oncogene, transcription factor	–0.06	0.000007
ZFP36L2	678	ZFP36 ring finger protein like 2	–0.51	0.000017
MIER3	166968	MIER family member 3	–0.13	0.000034
ZBTB5	9925	Zinc finger and BTB domain containing 5	–0.13	0.000091
HHEX	3087	Hematopoietically expressed homeobox	–0.40	0.000113
GTF2B	2959	General transcription factor IIB	–0.27	0.000117
DYRK2	8445	Dual specificity tyrosine phosphorylation regulated kinase 2	–0.18	0.000219
NFIA	4774	Nuclear factor I A	–0.07	0.000238
ZBTB39	9880	Zinc finger and BTB domain containing 39	–0.09	0.000260
NIF3L1	60491	NGG1 interacting factor 3 like 1	–0.17	0.000366
ZBTB10	65986	Zinc finger and BTB domain containing 10	–0.16	0.000424
PRMT6	55170	Protein arginine methyltransferase 6	–0.27	0.000430
TFB2M	64216	Transcription factor B2, mitochondrial	–0.18	0.000439
ASCC1	51008	Activating signal cointegrator 1 complex subunit 1	–0.13	0.000448
SAP30L	79685	Histone deacetylase complex subunit SAP30L	–0.17	0.000478
H2AFV	94239	H2A histone family member V	–0.19	0.000517
SMARCA2	6595	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	–0.32	0.000538
SRPRB	58477	SRP receptor beta subunit	–0.18	0.000574
KLF10	7071	Kruppel like factor 10	–0.26	0.000576
BRD3	8019	Bromodomain containing 3	–0.25	0.000713
PPARA	5465	Peroxisome proliferator activated receptor alpha	–0.14	0.000750
INTS7	25896	Integrator complex subunit 7	–0.14	0.000763
SESN3	143686	Sestrin 3	–0.27	0.000795
ZHX1	11244	Zinc fingers and homeoboxes 1	–0.28	0.000816
PARN	5073	Poly(A)–specific ribonuclease	–0.24	0.000862
SNUPN	10073	Snurportin 1	–0.11	0.000893
GTF2E1	2960	General transcription factor IIE subunit 1	–0.19	0.000962
LRRC32	2615	Leucine rich repeat containing 32	0.43	0.000983
RBM17	84991	RNA binding motif protein 17	–0.16	0.000983
NCBP1	4686	Nuclear cap binding protein subunit 1	–0.19	0.001140
ZNF717	100131827	Zinc finger protein 717	–0.07	0.001234
ZNF318	24149	Zinc finger protein 318	–0.16	0.001235

Gene Symbol	Gene ID	Gene Name	Log_2 FC	*p*-value
FLI1	2313	Fli-1 proto-oncogene, ETS transcription factor	–0.28	0.001262
OVOL1	5017	Ovo like transcriptional repressor 1	0.22	0.001286
ZNF107	51427	Zinc finger protein 107	–0.08	0.001293
IMMUNOREGULATION				
TNFAIP3	7128	TNF alpha induced protein 3	0.53	0.000004
GIMAP4	55303	GTPase, IMAP family member 4	–0.51	0.000008
SLAMF6	114836	SLAM family member 6	–0.12	0.000016
DAPP1	27071	Dual adaptor of phosphotyrosine and 3-phosphoinositides 1	–0.27	0.000025
MEF2C	4208	Myocyte enhancer factor 2C	–0.09	0.000045
PLAUR	5329	Plasminogen activator, urokinase receptor	0.32	0.000123
BST1	683	Bone marrow stromal cell antigen 1	–0.17	0.000160
NFKBIA	4792	NFKB inhibitor alpha	0.54	0.000181
PROS1	5627	Protein S (alpha)	–0.14	0.000317
MNDA	4332	Myeloid cell nuclear differentiation antigen	–0.42	0.000405
MLEC	9761	Malectin	–0.26	0.000457
METTL7A	25840	Methyltransferase like 7A	–0.45	0.000602
TLR5	7100	Toll-like receptor 5	–0.18	0.000637
MAP2K3	5606	Mitogen-activated protein kinase kinase 3	0.33	0.000702
LRMP	4033	Lymphoid restricted membrane protein	–0.25	0.000720
SRC	6714	SRC proto-oncogene, non-receptor tyrosine kinase	0.33	0.000721
DUSP4	1846	Dual specificity phosphatase 4	0.12	0.000786
SNAP29	9342	Synaptosome associated protein 29	–0.17	0.000790
ZC3HAV1	56829	Zinc finger CCCH-type containing, antiviral 1	–0.22	0.000833
IFI16	3428	Interferon gamma inducible protein 16	–0.38	0.000834
SH3BP2	6452	SH3 domain binding protein 2	0.22	0.000869
GAPT	202309	GRB2 binding adaptor protein, transmembrane	–0.16	0.000888
ALDOC	230	Aldolase, fructose-bisphosphate C	0.16	0.000975
LAIR1	3903	Leukocyte associated immunoglobulin like receptor 1	0.12	0.001022
ATF4	468	Activating transcription factor 4	0.17	0.001146
METABOLISM				
C9orf64	84267	Chromosome 9 open reading frame 64	–0.22	0.000037
HPGD	3248	Hydroxyprostaglandin dehydrogenase 15-(NAD)	–0.09	0.000053
TIMMDC1	51300	Translocase of inner mitochondrial membrane domain containing 1	–0.15	0.000123
ICK	22858	Intestinal cell kinase	–0.17	0.000213
DDO	8528	D-aspartate oxidase	–0.20	0.000255

Gene Symbol	Gene ID	Gene Name	Log_2 FC	*p*-value
DCTD	1635	dCMP deaminase	–0.15	0.000257
CDYL2	124359	Chromodomain Y like 2	–0.12	0.000312
GLRX	2745	Glutaredoxin	–0.15	0.000313
MFSD2A	84879	Major facilitator superfamily domain containing 2A	0.54	0.000317
TPK1	27010	Thiamin pyrophosphokinase 1	–0.17	0.000325
PDPR	55066	Pyruvate dehydrogenase phosphatase regulatory subunit	–0.14	0.000338
SPTLC2	9517	Serine palmitoyltransferase long chain base subunit 2	–0.31	0.000344
PDK4	5166	Pyruvate dehydrogenase kinase 4	–0.54	0.000400
HCCS	3052	Holocytochrome c synthase	–0.22	0.000431
SPTSSA	171546	Serine palmitoyltransferase small subunit A	–0.33	0.000463
FAR2	55711	Fatty acyl-CoA reductase 2	–0.32	0.000470
HERPUD1	9709	Homocysteine inducible ER protein with ubiquitin like domain 1	–0.27	0.000472
CLYBL	171425	Citrate lyase beta like	–0.06	0.000506
KLF4	9314	Kruppel like factor 4	–0.30	0.000506
INSIG1	3638	Insulin induced gene 1	1.13	0.000533
ALDH3A2	224	Aldehyde dehydrogenase 3 family member A2	–0.28	0.000534
EPM2A	7957	EPM2A, laforin glucan phosphatase	–0.09	0.000562
INPP1	3628	Inositol polyphosphate-1-phosphatase	0.10	0.000587
EXTL2	2135	Exostosin like glycosyltransferase 2	–0.22	0.000598
CLPX	10845	Caseinolytic mitochondrial matrix peptidase chaperone subunit	–0.26	0.000635
ACOX1	51	Acyl-CoA oxidase 1	–0.20	0.000644
ALDH5A1	7915	Aldehyde dehydrogenase 5 family member A1	–0.17	0.000705
THRAP3	9967	Thyroid hormone receptor associated protein 3	–0.19	0.000723
DIS3L	115752	DIS3 like exosome 3’-5’ exoribonuclease	–0.14	0.000792
TMLHE	55217	Trimethyllysine hydroxylase, epsilon	–0.18	0.000814
ENO2	2026	Enolase 2	0.38	0.000828
ERI2	112479	ERI1 exoribonuclease family member 2	–0.22	0.000845
SCO1	6341	SCO1, cytochrome c oxidase assembly protein	–0.13	0.000859
EPRS	2058	Glutamyl-prolyl-tRNA synthetase	–0.25	0.000874
GLRX2	51022	Glutaredoxin 2	–0.23	0.000911
PARP9	83666	Poly(ADP-ribose) polymerase family member 9	–0.21	0.000996
GNPDA2	132789	Glucosamine-6-phosphate deaminase 2	–0.09	0.001026
GTF2I	2969	General transcription factor IIi	–0.17	0.001057
DNAJC16	23341	DnaJ heat shock protein family (Hsp40) member C16	–0.17	0.001065

Gene Symbol	Gene ID	Gene Name	Log_2 FC	*p*-value
STARD7	56910	StAR related lipid transfer domain containing 7	–0.34	0.001099
FIG4	9896	FIG4 phosphoinositide 5-phosphatase	–0.28	0.001105
LIAS	11019	Lipoic acid synthetase	–0.18	0.001124
ACP6	51205	Acid phosphatase 6, lysophosphatidic	–0.06	0.001225
MOLECULE TRANSPORT				
ERLIN1	10613	ER lipid raft associated 1	–0.25	0.000016
SLC10A7	84068	Solute carrier family 10 member 7	–0.17	0.000169
UNC50	25972	UNC-50 inner nuclear membrane RNA binding protein	–0.26	0.000354
ATP10D	57205	TPase phospholipid transporting 10D (putative)	–0.16	0.000491
SLC40A1	30061	Solute carrier family 40 member 1	–0.23	0.000640
SLC43A3	29015	Solute carrier family 43 member 3	0.23	0.000646
CLCN3	1182	Chloride voltage-gated channel 3	–0.37	0.000672
SLCO4A1	28231	Solute carrier organic anion transporter family member 4A1	0.56	0.000724
CLCN6	1185	Chloride voltage-gated channel 6	0.20	0.000767
STIM2	57620	Stromal interaction molecule 2	–0.22	0.000993
ATP2A2	488	ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2	–0.20	0.001067
SLC30A1	7779	Solute carrier family 30 member 1	–0.52	0.001132
TMEM184C	55751	Transmembrane protein 184C	–0.19	0.001167
SLC1A3	6507	Solute carrier family 1 member 3	0.20	0.001252
NON-CODING PROTEIN GENES				
AC009506.1	643072	Uncharacterised LOC643072	–0.21	0.000014
AC093323.3	93622	Morf4 Family Associated Protein 1 Like 1 Pseudogene (LOC93622)	–0.14	0.000740
CARD8-AS1	100505812	CARD8 antisense RNA 1	–0.44	0.000078
DHRS4-AS1	55449	DHRS4 antisense RNA 1	–0.17	0.000620
GGTA1P	2681	Glycoprotein, alpha-galactosyltransferase 1 pseudogene	–0.25	0.000928
MIR762HG	101928736	MIR762 host gene	–0.08	0.000437
RP11-465B22.3	100288175	Uncharacterised LOC100288175	0.21	0.000555
RP11-506M13.3	101929506	Uncharacterised LOC101929506	–0.09	0.000694
ZNF542P	147947	Zinc finger protein 542, pseudogene	–0.12	0.000113
OTHER PROCESSES				
ABHD17B	51104	Abhydrolase domain containing 17B	–0.26	0.000158
C11orf54	28970	Chromosome 11 open reading frame 54	–0.19	0.000568

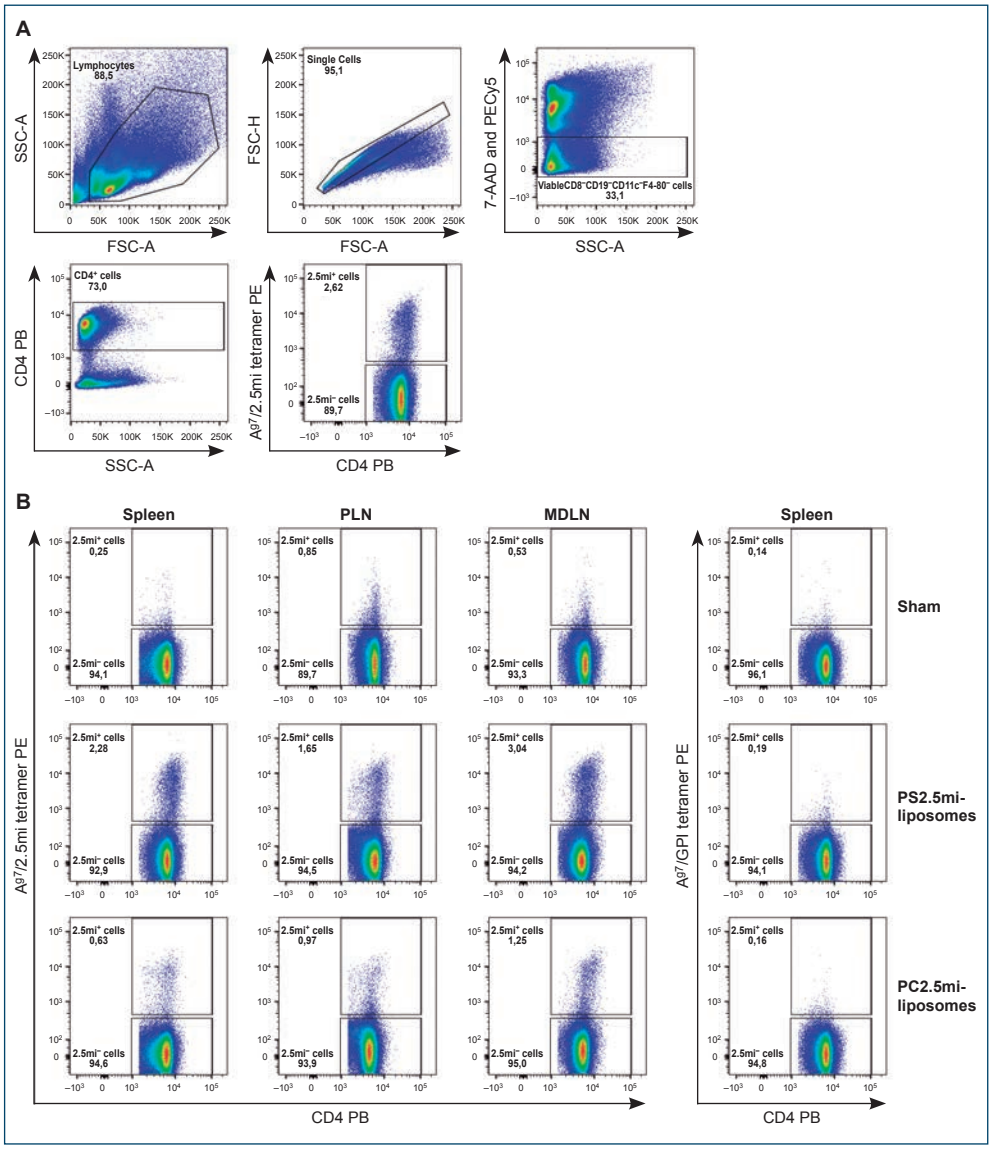
Gene Symbol	Gene ID	Gene Name	Log_2 FC	*p*-value
C5orf30	90355	Chromosome 5 open reading frame 30	–0.14	0.000179
FAM118B	79607	Family with sequence similarity 118 member B	–0.22	0.000029
HERPUD2	64224	HERPUD family member 2	–0.23	0.000162
SPIN3	169981	Spindlin family member 3	–0.11	0.001076
TRAF3IP1	26146	TRAF3 interacting protein 1	–0.12	0.000580
POST-TRANSLATIONAL PROTEIN MODIFICATION				
PPME1	51400	Protein phosphatase methylesterase 1	0.13	0.000062
FBXO36	130888	F-box protein 36	–0.11	0.000192
NSMCE4A	54780	NSE4 homolog A, SMC5-SMC6 complex component	–0.18	0.000419
VWA5A	4013	Von Willebrand factor A domain containing 5A	–0.21	0.000616
FBXO25	26260	F-box protein 25	–0.08	0.000669
DCAF12	25853	DDB1 and CUL4 associated factor 12	–0.27	0.000672
CBX4	8535	Chromobox 4	–0.21	0.000704
RMND5A	64795	Required for meiotic nuclear division 5 homolog A	–0.34	0.000710
LNX2	222484	Ligand of numb-protein X 2	–0.15	0.000821
BTBD3	22903	BTB domain containing 3	–0.21	0.000926
TMEM5	10329	Transmembrane protein 5	–0.11	0.000987
PREP	5550	Prolyl endopeptidase	–0.18	0.001025
KCTD2	23510	Potassium channel tetramerisation domain containing 2	–0.25	0.001056
SIGNAL TRANSDUCTION				
SNN	8303	Stannin	–0.43	0.000007
SHB	6461	SH2 domain containing adaptor protein B	0.31	0.000026
SKI	6497	SKI proto-oncogene	–0.26	0.000069
PAQR8	85315	Progestin and adipoQ receptor family member 8	–0.38	0.000160
UBFD1	56061	Ubiquitin family domain containing 1	–0.10	0.000209
N4BP1	9683	NEDD4-binding protein 1	–0.20	0.000221
FZD5	7855	Frizzled class receptor 5	–0.07	0.000329
NET1	10276	Neuroepithelial cell transforming 1	–0.23	0.000369
ZBED3	84327	Zinc finger BED-type containing 3	–0.21	0.000615
FRAT2	23401	FRAT2, WNT signalling pathway regulator	–0.41	0.000675
PEX11B	8799	Peroxisomal biogenesis factor 11 beta	–0.19	0.000726
TFDP2	7029	Transcription factor Dp-2	–0.12	0.000787
DACT1	51339	Dishevelled binding antagonist of beta catenin 1	–0.16	0.000831
RGS18	64407	Regulator of G protein signalling 18	–0.38	0.001044
MAP4K4	9448	Mitogen-activated protein kinase kinase kinase kinase 4	–0.12	0.001103

Gene Symbol	Gene ID	Gene Name	Log_2 FC	*p*-value
ARHGAP25	9938	Rho GTPase activating protein 25	–0.17	0.001144
AHR	196	Aryl hydrocarbon receptor	–0.57	0.001222
ABHD2	11057	Abhydrolase domain containing 2	–0.45	0.001298
UNCHARACTERISED				
C21orf91	54149	Chromosome 21 open reading frame 91	–0.21	0.000813
EVI2B	2124	Ecotropic viral integration site 2B	–0.60	0.000405
FAM102B	284611	Family with sequence similarity 102 member B	–0.26	0.000813
FAM105A	54491	Family with sequence similarity 105 member A	–0.33	0.001104
FAM120C	54954	Family with sequence similarity 120C	–0.07	0.000614
FAM217B	63939	Family with sequence similarity 217 member B	–0.19	0.000825
PGBD2	267002	PiggyBac transposable element derived 2	–0.12	0.000096
TMEM248	55069	Transmembrane protein 248	–0.16	0.000560
TRANK1	9881	Tetratricopeptide repeat and ankyrin repeat containing 1	–0.14	0.000779
YPEL2	388403	Yippee like 2	–0.31	0.000874
VESICLE-MEDIATED PROCESSES				
CYTH4	27128	Cytohesin 4	0.31	0.000013
GOLPH3L	55204	Golgi phosphoprotein 3 like	–0.28	0.000084
SEC22C	9117	SEC22 homolog C, vesicle trafficking protein	–0.14	0.000341
LDLR	3949	Low density lipoprotein receptor	0.80	0.000672
RAB32	10981	RAB32, member RAS oncogene family	–0.17	0.000762
EHBP1	23301	EH domain binding protein 1	–0.08	0.000840
KIF20B	9585	Kinesin family member 20B	–0.15	0.000851
SNX18	112574	Sorting nexin 18	–0.37	0.001208

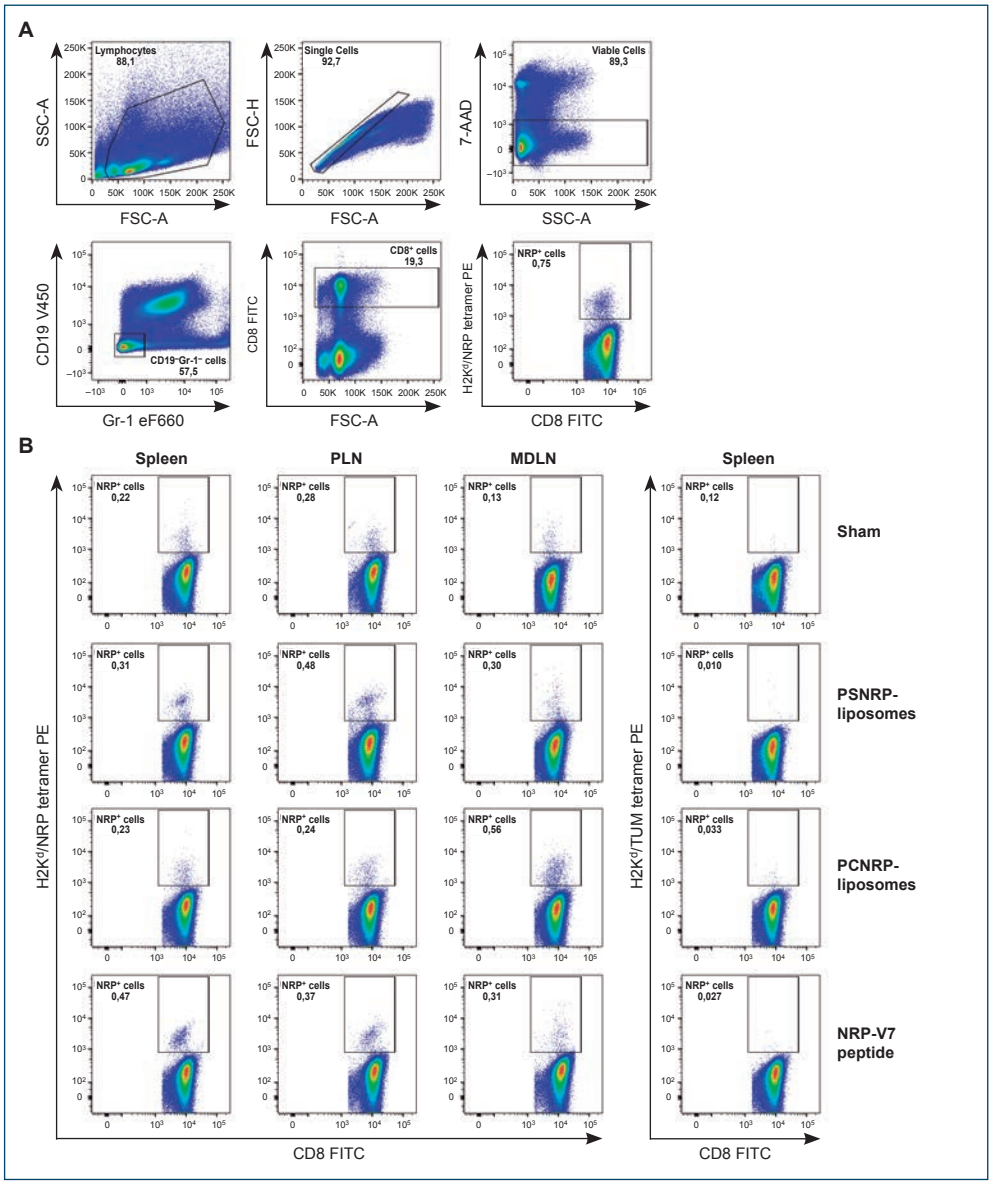
Supplementary Table 2. Correlation between AUC from PSOG488-liposomes capture kinetics by DCs from paediatric patients with T1D and clinical data of the same patients

Correlation	Spearman r	Spearman *p*
AUC vs Age (years)	–0.2198	0.4703
AUC vs BMI (kg/m^2)	–0.2311	0.4441
AUC vs Age at T1D diagnosis (years)	0.03297	0.9205
AUC vs HbA1c (%)	0.489	0.0930
AUC vs Fasting C-peptide (ng/mL)	0.05798	0.9333
AUC vs Insulin dose (IU/kg/day)	0.1436	0.6410

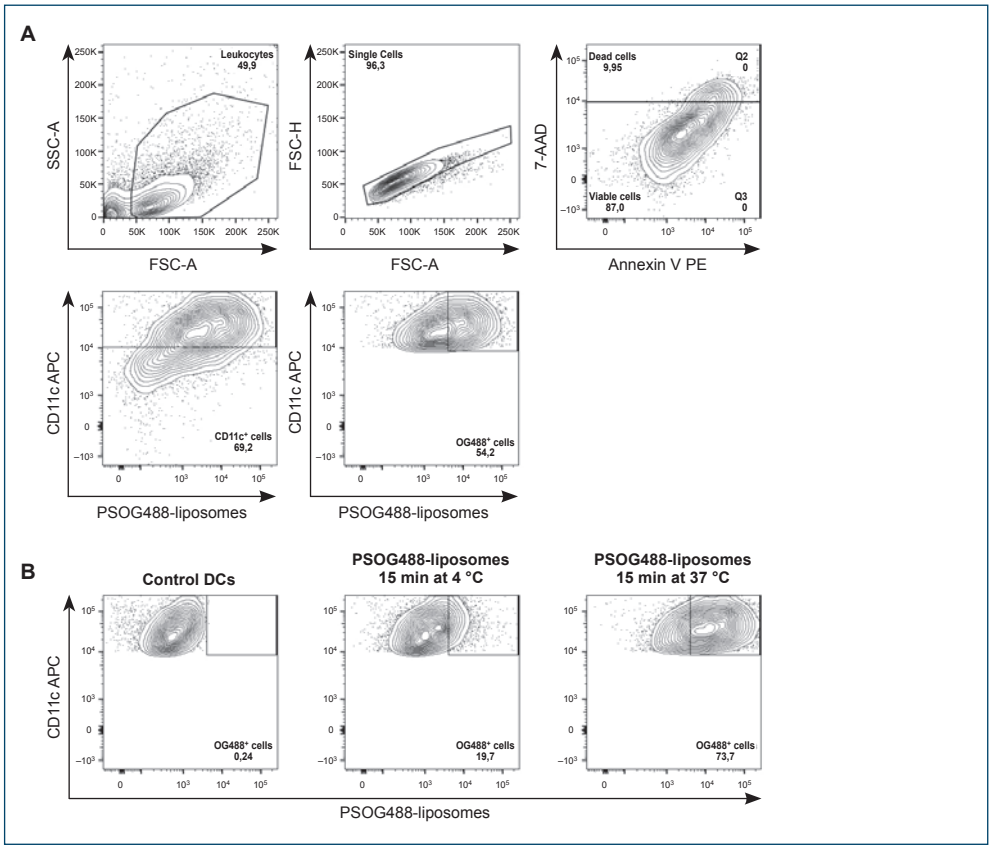
Spearman's r and *p*-value calculated from Spearman's correlation analysis. BMI: body mass index; T1D: type 1 diabetes; HbA1c: glycated haemoglobin.



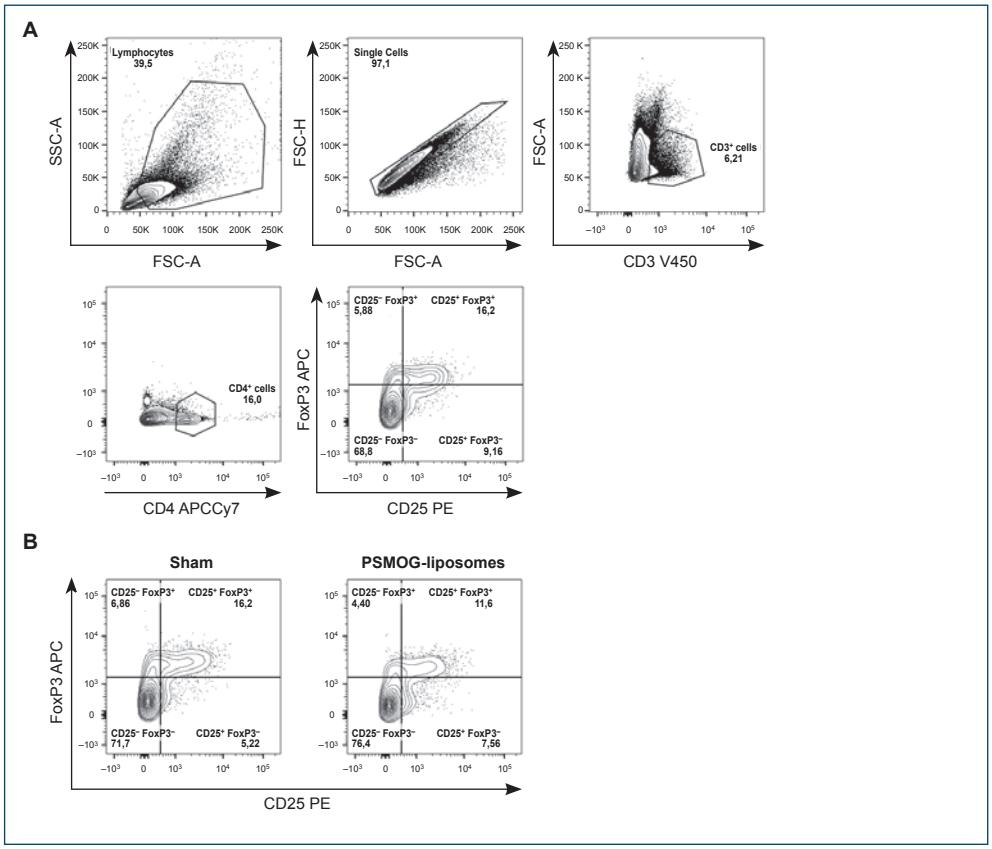
Supplementary Figure 1. ***Flow cytometry analysis of 2.5mi⁺CD4⁺ T cells expanded 4 days after i.p. administration of PS2.5mi-liposomes to NOD mice.*** **(A)** *Gating strategy used to analyse the expansion of 2.5mi⁺CD4⁺ T cells after the administration of PS2.5mi-liposomes. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H). Dead cells along with CD8⁺, CD19⁺, CD11c⁺ and F4/80⁺ cells were excluded with the use of 7-AAD, CD8 PECy5, CD19 PECy5, CD11c PECy5 and F4/80 PECy5. Positive cells for CD4 PB stain were selected, and the percentage of positive and negative cells for A^{g7}/2.5mi tetramer PE was gated. Shown is a representative gating strategy of splenocytes obtained from an animal treated with PS2.5mi-liposomes.* **(B)** *Representative flow cytometry pseudocolour plots showing the percentage of tetramer⁺ T cells and tetramer⁻ T cells in the several treated groups and secondary lymphoid organs. Left panel: A^{g7}/2.5mi tetramer⁺ and A^{g7}/2.5mi tetramer⁻ (gated in CD4⁺) T cells in spleen, PLN and MDLN from sham group, PS2.5mi-liposomes group and PC2.5mi-liposomes group. Right panel: control staining with A^{g7}/GPI tetramer in the spleen of the different treated groups.*



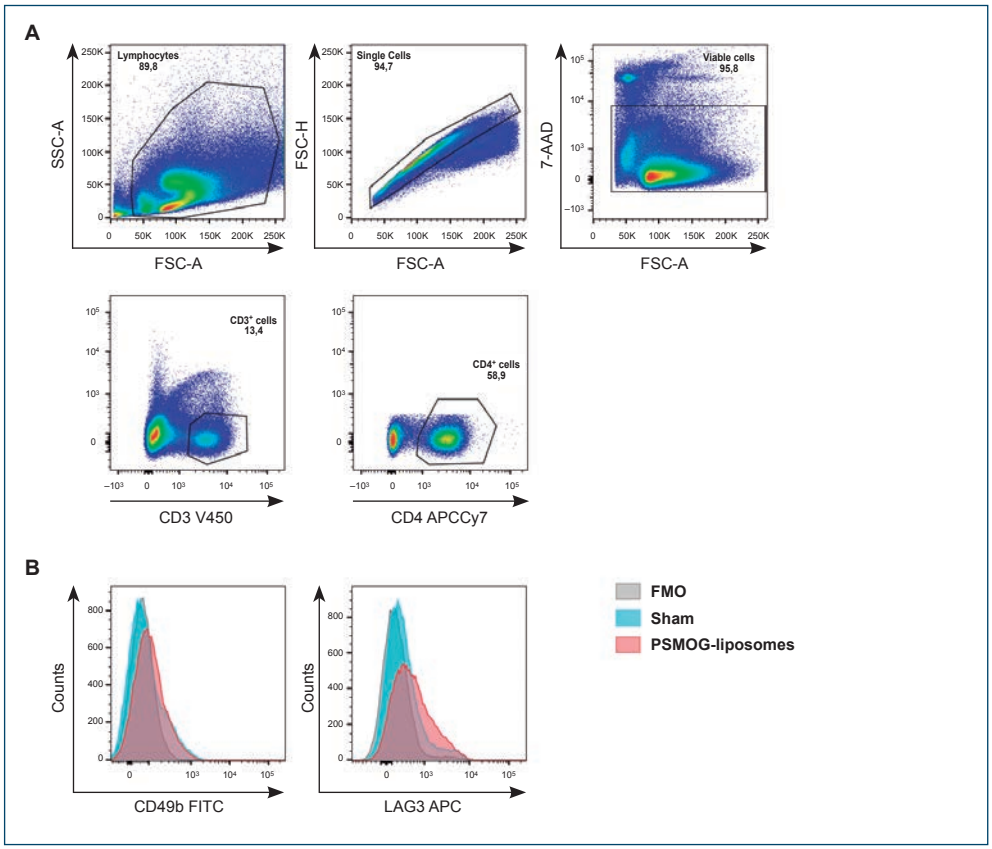
Supplementary Figure 2. ***Flow cytometry analysis of NRP⁺CD8⁺ T cells expanded 4 days after i.p. administration of PSNRP-liposomes to NOD mice.*** **(A)** *Gating strategy used to analyse the expansion of NRP⁺CD8⁺ T cells after the administration of PSNRP-liposomes. Cells were gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H). Dead cells were excluded with 7-AAD staining, and B cells and granulocytes were also excluded with the use of Gr-1 eF660 and CD19 V450. Positive cells for CD8 FITC stain were selected, and the percentage of positive cells for H2K^d/NRP tetramer PE was gated. Shown is a representative gating strategy of splenocytes from PSNRP-liposomes group.* **(B)** *Representative flow cytometry pseudocolour plots showing the percentage of tetramer⁺ T cells in the several treated groups and secondary lymphoid organs. Left panel: H2K^d/NRP tetramer⁺ (gated in CD8⁺) T cells in spleen, PLN and MDLN from sham, PSNRP-liposomes, PCNRP-liposomes and NRP-V7 peptide groups. Right panel: control staining with H2K^d/TUM tetramer (gated in CD8⁺) in the spleen of the different treated groups.*



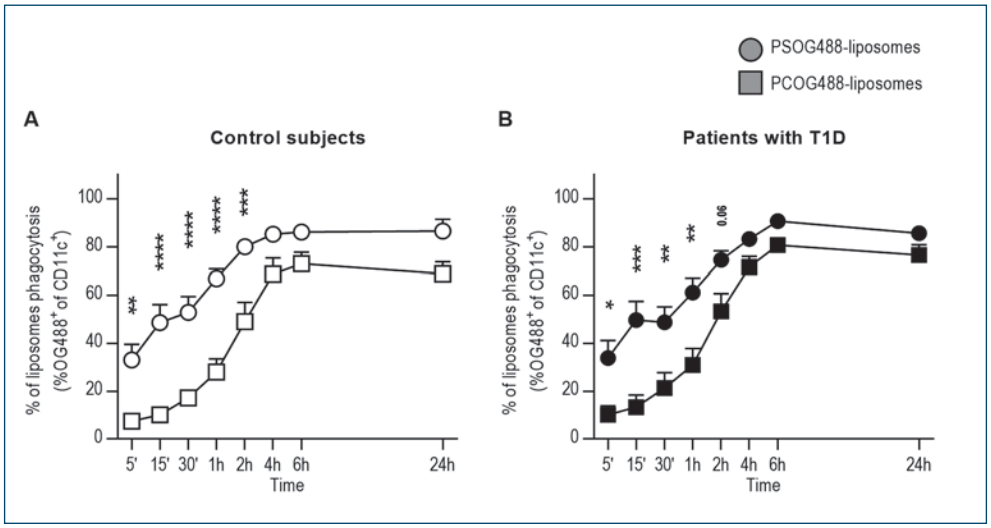
Supplementary Figure 3. ***Flow cytometry analysis of PS-liposomes capture kinetics by murine DCs.*** **(A)** *Gating strategy used to analyse the kinetics of empty fluorescent PSOG488-liposomes uptake (OG488⁺) by DCs (CD11c⁺) obtained from bone marrow precursor cells from EAE-induced C57BL/6 mice. Cells were first gated for leukocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H). Dead cells were excluded with the use of 7-AAD and Annexin V PE. Positive cells for CD11c APC stain were selected, and the percentage of positive cells for OG488 staining (meaning DCs that have captured PSOG488-liposomes) were gated. Shown is a representative gating strategy of DCs cultured with PSOG488-liposomes for 5 minutes at 37 °C.* **(B)** *Flow cytometry contour plots displaying the kinetics of PSOG488-liposomes capture. From left to right: DCs cultured without PSOG488-liposomes (control DCs), DCs cultured with PSOG488-liposomes for 15 minutes at 4 °C and at 37 °C.*



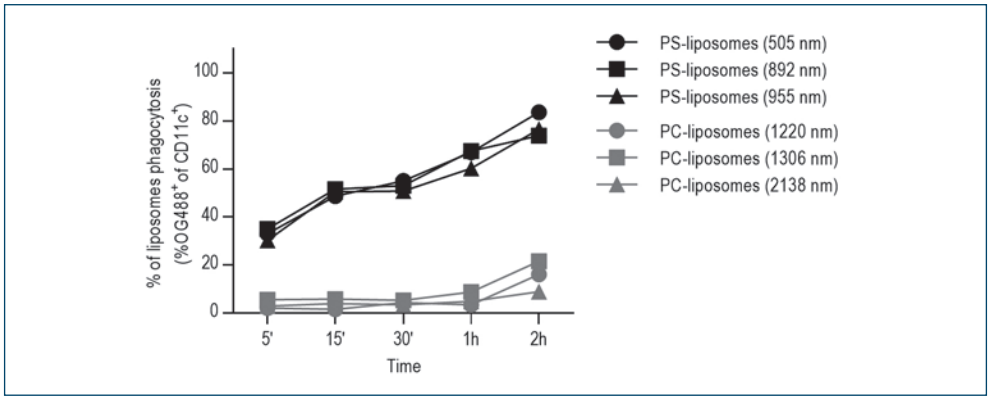
Supplementary Figure 4. ***Flow cytometry analysis of CD25⁺FoxP3⁺ regulatory and CD25⁺FoxP3⁻ T cells 6 days after administration of PSMOG-liposomes to EAE-induced mice.*** **(A)** *Gating strategy used to analyse the percentages of CD25⁺FoxP3⁺ and CD25⁺FoxP3⁻ T cells within CD3⁺CD4⁺ splenocytes. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H). Positive cells for CD3 V450 stain were selected, and then CD4 APCCy7 positive cells were gated. Finally, cells were divided into four quadrants according to the expression of CD25 and FoxP3 with the use of CD25 PE and FoxP3 APC antibodies. FMO controls were used to set the axis. Shown is a representative gating strategy of splenocytes obtained from an EAE-induced C57BL/6 mouse treated with PSMOG-liposomes.* **(B)** *Representative flow cytometry contour plots showing CD25⁺FoxP3⁺ and CD25⁺FoxP3⁻ T cells (gated within CD3⁺CD4⁺ cells) in the spleen of EAE-induced C57BL/6 mice treated with PBS (sham group, left panel) and with PSMOG-liposomes (PSMOG-liposomes group, right panel).*



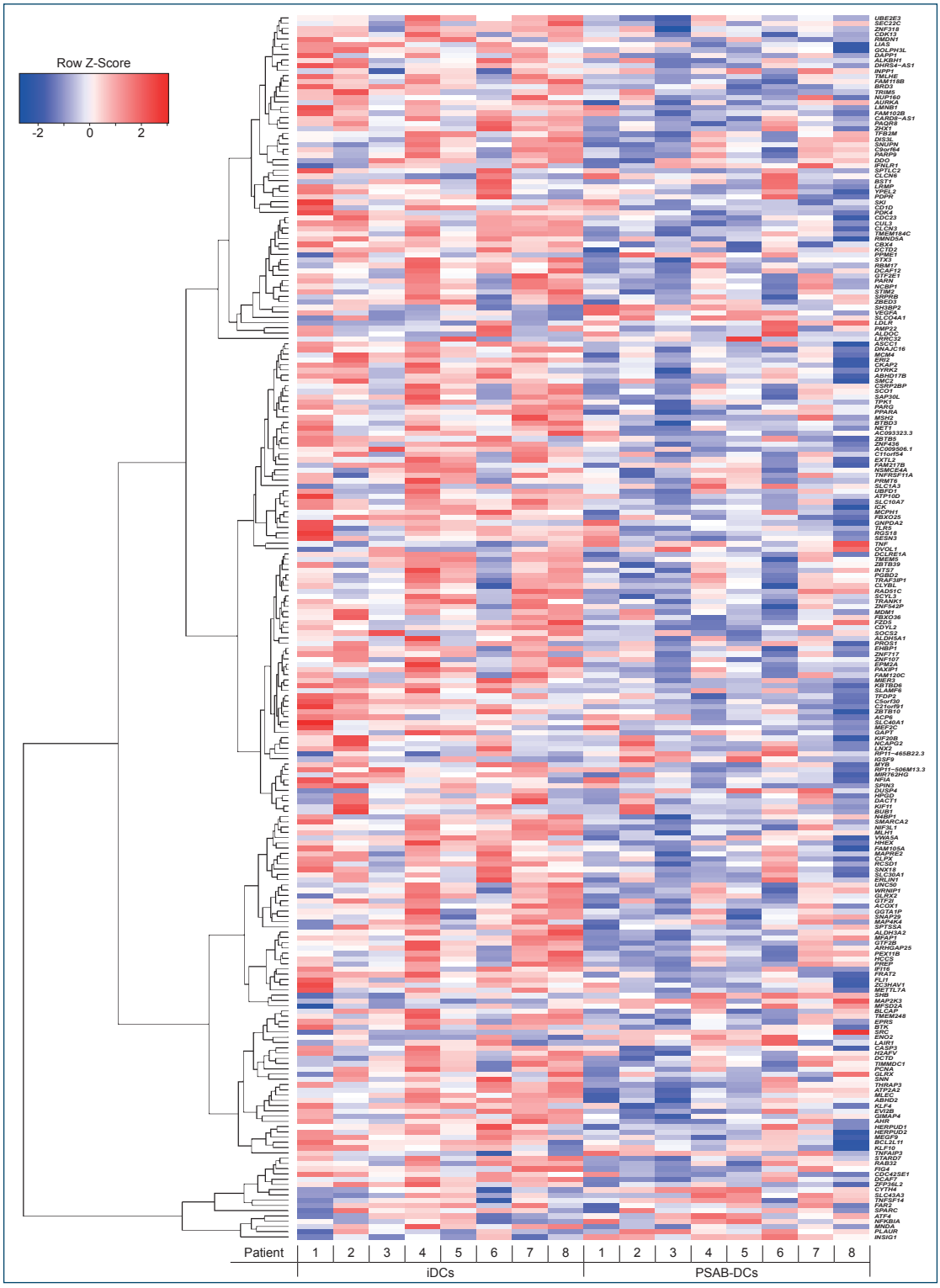
Supplementary Figure 5. ***Flow cytometry analysis of CD49b and LAG3 markers expression in CD4⁺ T cells 6 days after administration of PSMOG-liposomes to EAE-induced mice.*** **(A)** *Gating strategy used to analyse the MFI of CD49b and LAG3 markers in CD3⁺CD4⁺ splenocytes. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and singlets (FSC-A vs FSC-H). Dead cells were excluded with the use of 7-AAD, and viable cells were gated for CD3 positivity with the use of CD3 V450. Then, CD4 APCCy7 positive cells were gated. Shown is a representative pseudocolour gating strategy of splenocytes obtained from an EAE-induced C57BL/6 mouse treated with PSMOG-liposomes.* **(B)** *Histogram of one representative experiment for CD49b expression (left) and LAG3 expression (right) in mice treated with PBS (sham, blue) and PSMOG-liposomes (red). The corresponding FMO control is shown in grey.*



Supplementary Figure 6. ***Time course of PS-liposomes and PC-liposomes phagocytosis kinetics by DCs from adult control subjects and patients with T1D.*** **(A)** *Time course of the capture of fluorescently-labelled PSOG488-liposomes (circles) and PCOG488-liposomes (squares), performed by DCs from control subjects (white symbols, n=5) at 37 °C. Results are mean ± SEM. Comparison between PS-liposomes and PC-liposomes phagocytosis at different time points showed significant differences (**p<0.01, ***p<0.001, ****p<0.0001, Two-way ANOVA).* **(B)** *Time course of the capture of fluorescently-labelled PSOG488-liposomes (circles) and PCOG488-liposomes (squares), performed by DCs from patients with T1D (black symbols, n=10) at 37 °C. Results are mean ± SEM. Comparison between PS-liposomes and PC-liposomes phagocytosis at different time points showed significant differences (*p<0.05, **p<0.01, ***p<0.001, Two-way ANOVA).*

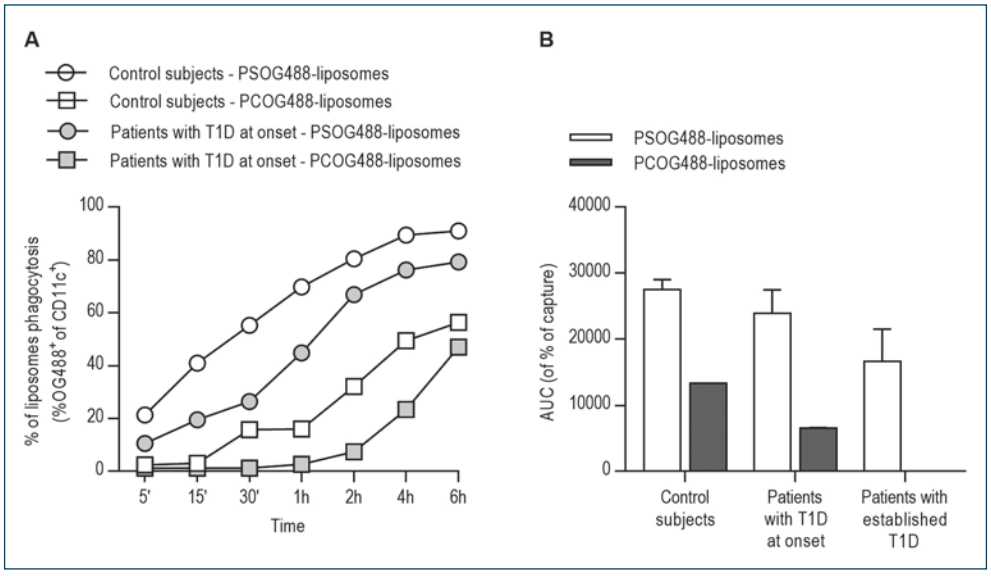


Supplementary Figure 7. ***Time course of phagocytosis of PS-liposomes and PC-liposomes according to particle size.*** *Uptake of fluorescently-labelled (OG488) liposomes performed by DCs from one control subject at 37 °C. Black lines indicate phagocytosis of empty fluorescent PSOG488-liposomes with 505 nm (circles), 892 nm (squares) and 955 nm (triangles) of diameter; grey lines show empty fluorescent PS-free PCOG488-liposomes with 1,220 nm (circles), 1,306 nm (squares) and 2,138 nm (triangles) of diameter.*

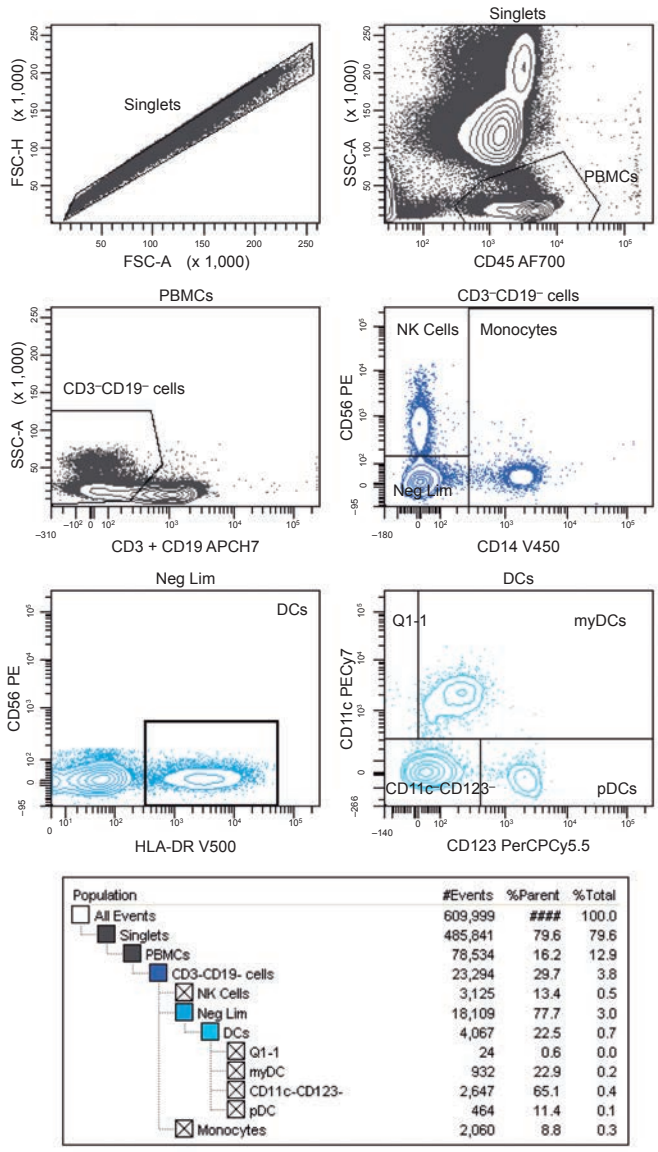


Supplementary Figure 8. *Continued*

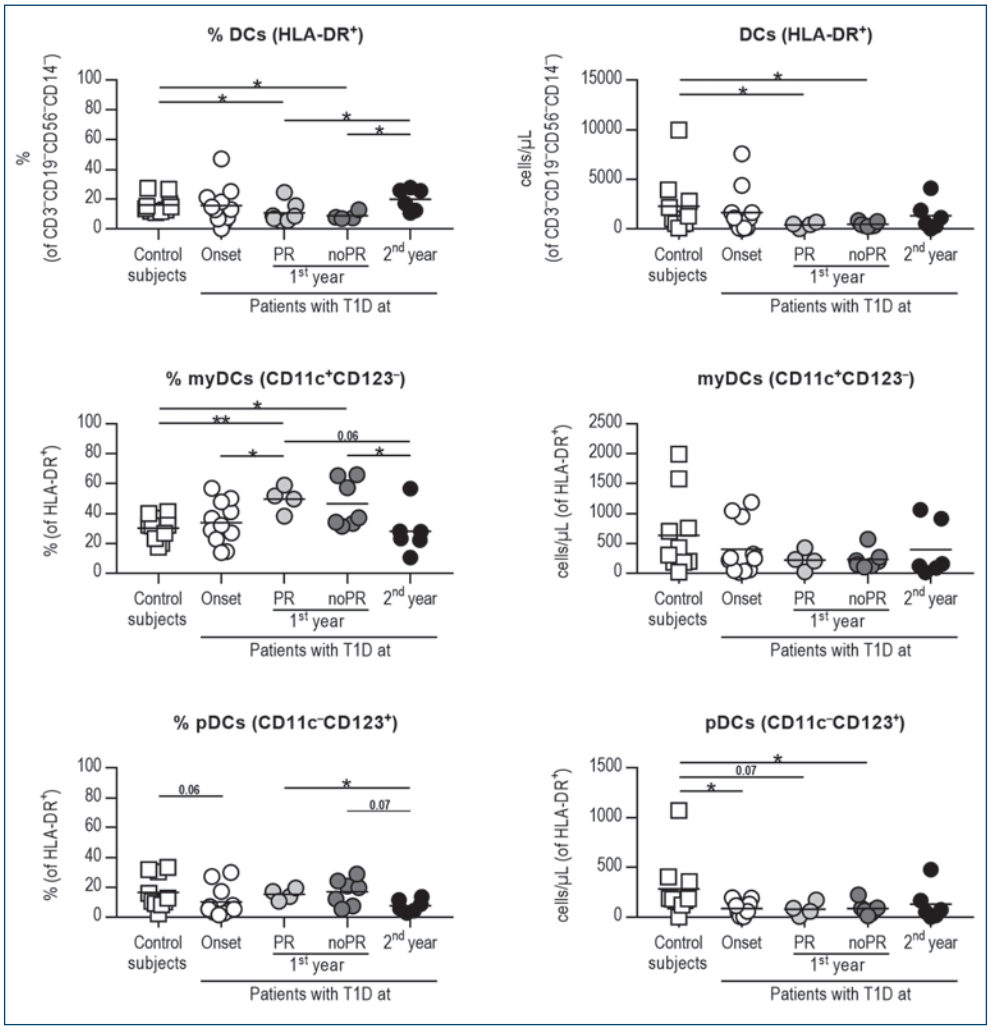
Supplementary Figure 8. ***Heatmap of the DEGs in DCs from 8 patients with T1D after phagocytosis of PSAB-liposomes.*** *Heatmap of the 233 genes considered as DEG (p-value<0.0013, adjusted p-value<0.1254, and Log_2 of FC >0.05 or <–0.05) resulting from the RNA-seq analysis. The first 8 columns (on the right) represent individual samples of iDCs obtained from 8 patients with T1D, whereas the left 8 columns represent DCs after the phagocytosis of PSAB-liposomes (PSAB-DCs) from the same patients. The rows show specific genes of interest. The Row-Z score of each gene measures deviation from its mean, and it is depicted by colour intensity, with blue indicating downregulated and red indicating upregulated expression.*



Supplementary Figure 9. ***Time course of PS-liposomes and PC-liposomes phagocytosis kinetics by DCs from paediatric control subjects and patients with T1D at onset.*** **(A)** *Time course of the capture of fluorescently-labelled PSOG488-liposomes (circles) and PCOG488-liposomes (squares), performed by DCs from control subjects (n=1, white symbols) and patients with T1D at onset (n=2, grey symbols) at 37 °C. Results are mean ± SEM.* **(B)** *AUC of phagocytosis kinetics curves of PSOG488-liposomes (white bars) and PCOG488-liposomes (dark grey bars). Bars display the mean ± SD of results from control subjects (n=6 in PSOG488-liposomes condition and n=1 in PCOG488-liposomes condition), paediatric patients with T1D at onset (n=6 in PSOG488-liposomes condition and n=2 in PCOG488-liposomes condition), and patients with T1D with established disease (n=7 in PSOG488-liposomes condition). Differences were not found when comparing PSOG488-liposomes and PCOG488-liposomes conditions in each group.*



Supplementary Figure 10. ***Flow cytometry analysis of DCs subsets in peripheral blood of paediatric subjects with T1D.*** *Gating strategy used to analyse the percentages and absolute counts of the different DCs subsets. Cells were first gated for singlets (FSC-A vs FSC-H), and then PBMCs with the use of CD45 AF700 (CD45⁺ vs SSC-A). Afterwards, cells were gated for CD3 and CD19 negativity with the use of CD3 APCH7 and CD19 APCH7. NK cells and monocytes were excluded with the use of CD56 PE and CD14 V450, respectively, and DCs were gated with the use of HLA-DR V500. Finally, the different subsets of DCs were analysed using CD123 PerCPCy5.5 and CD11c PECy7, thus revealing 3 subsets: myDCs (CD11c⁺CD123⁻), pDCs (CD11c⁻CD123⁺) and a non-categorised subset that is CD11c⁻CD123⁻. Shown is a representative gating strategy and contour plots of peripheral blood DCs obtained from a paediatric patient at 2nd year of evolution.*



Supplementary Figure 11. ***Percentages and numbers of DCs, myDCs and pDCs are similar between patients undergoing PR and those not undergoing PR at 1st year of evolution of the disease.*** *Percentages (left panel) and concentration (cells/µL) of total DCs (1st row, HLA-DR+ of CD3-CD19-CD56-CD14- cells), myDCs (2nd row, CD11c+CD123- of HLA-DR+ cells) and pDCs (3rd row, CD11c-CD123+ of HLA-DR+ cells). White squares represent control subjects (n=10), and circles represent paediatric patients with T1D at different stages of the disease: onset (n=11), white; undergoing PR at 1st year of evolution (n=4, PR), light grey; not undergoing PR at 1st year of evolution (n=7, noPR), dark grey, and at 2nd year of evolution (n=11), black. Statistically significant differences were found when comparing the groups (*p<0.05, **p<0.01, Mann-Whitney test).*

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As of today, breaches of immunological tolerance cannot be prevented or reversed permanently. In physiological conditions, clearance of apoptotic cells by phagocytes triggers tolerogenic signalling, hinders autoimmune recognition and maintains homeostasis; three critical processes to tackle an autoimmune disease. To that end, an original way to synthetically mimic apoptotic cells is to generate liposomes enriched in phosphatidylserine —a known apoptotic cell signal— and loaded with autoantigenic peptides. These liposomes have arrested experimental type 1 diabetes through the generation of tolerogenic dendritic cells. But how does this treatment successfully restore tolerance? Could it work in human disease? Is it applicable to other antigen-driven autoimmune diseases? In this work, we aimed at globally characterising the potential of this phosphatidylserine-rich liposomal strategy. Although further research is essential, this immunotherapy achieves apoptotic mimicry in a simple, safe and efficient manner, and poses as a potentially game-changing approach to recover antigen-specific tolerance in several autoimmune diseases.

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