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Targeting macrophages with phosphatidylserine-rich liposomes as a potential antigen-specific immunotherapy for type 1 diabetes

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A B S T R A C T

Type 1 diabetes (T1D) results from a breakdown in immunological tolerance, with pivotal involvement of antigen-presenting cells. In this context, antigen-specific immunotherapies have been developed to arrest autoimmunity, such as phosphatidylserine (PS)-liposomes. However, the role of certain antigen-presenting cells in immunotherapy, particularly human macrophages (Mφ) in T1D remains elusive. The aim of this study was to determine the role of Mφ in antigen-specific immune tolerance and T1D. To that end, we evaluated Mφ ability to capture apoptotic-body mimicking PS-liposomes in mice and conducted a phenotypic and functional characterisation of four human monocyte-derived Mφ (MoMφ) subpopulations (M0, M1, M2a and M2c) after PS-liposomes uptake. Our findings in mice identified Mφ as the most phagocytic cell subset in the spleen and liver. In humans, while phagocytosis rates were comparable between T1D and control individuals, PS-liposome capture dynamics differed among Mφ subtypes, favouring inflammatory (M1) and deactivated (M2c) Mφ. Notably, high nanoparticle concentrations did not affect macrophage viability. PS-liposome uptake by Mφ induced alterations in membrane molecule expression related to immunoregulation, reduced secretion of IL-6 and IL-12, and diminished autologous T-cell proliferation in the context of autoantigen stimulation. These results underscore the tolerogenic effects of PS-liposomes and emphasize their potential to target human Mφ, providing valuable insights into the mechanism of action of this preclinical immunotherapy.

1. Introduction

Since the identification of T1D as an autoimmune disease in 1974, efforts have focused on identifying the immunological events that trigger the destruction of insulin-producing β-cells in the pancreatic islets [1,2]. The mechanisms that precede the onset of the disease imply interaction between genetic and environmental factors that cause β-cells malfunction and the subsequent loss of tolerance towards islet-associated antigens (e.g., insulin, glutamic acid decarboxylase-65, Tyrosine-phosphatase 2 protein, etc.) which leads to β-cell destruction by autoreactive T cells, mainly $CD8^+$ [3].

Despite significant progress in understanding the disease, exogenous insulin administration remains the unique therapeutic option for T1D. While insulin replacement therapy significantly improves the patient's quality of life, it fails to prevent long-term secondary complications. Notably, the lack of curative treatments persists, with recent advances leaning towards therapies that aim to prevent but do not cure, exemplified by Teplizumab [4]. This highlights the urgent clinical need for the development of curative and safe therapies for T1D, a need shared by other autoimmune diseases. In this context, antigen-specific immunotherapies (ASITs) emerge as a promising approach to selectively target the cause of the disease [5], the pathogenic adaptive immune response against islet-associated antigens.

In this context, we have previously demonstrated the efficacy of an immunotherapy that mimicks apoptotic bodies –phosphatidylserine (PS)-liposomes– in addressing various autoimmune diseases including T1D [6–8]. PS-liposomes utilize efferocytosis, a conserved mechanism of apoptotic cell clearance, to induce immune tolerance. During apoptosis, PS –a phospholipid found in the inner leaflet of the lipid bilayer– translocates to the outer leaflet and interacts with phagocytes, enforcing

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a tolerogenic message. Subsequently, antigen-presenting cells (APCs) present the autoantigens from engulfed apoptotic cells in a regulatory manner, thereby promoting self-tolerance [9].

The development of most therapeutic ASITs involves *in vivo* targeting of APCs. These cells, upon engagement with therapeutic particles, undergo tolerogenic changes and present the delivered antigens in Human Leukocyte Antigens (HLA) complexes. This process aims to induce robust, safe and long-lasting tolerance [10]. However while this generally accepted mechanism of action holds promise, its efficacy may be compromised by observed alterations in myeloid cells in individuals with T1D, such as abnormal levels of phenotypic markers in peripheral blood dendritic cells (DCs) [11], aberrant cytokine secretion in monocytes and macrophages [12,13] or increased activation of nuclear factor (NF)-κB [14].

Until now, many studies discussed the role of Mφ T1D, but most of them have been conducted in mice [15–17]. Their involvement in self-tolerance induction after administering an immunotherapy in human T1D is not fully understood. An in-depth understanding of this cell subset in T1D is crucial for its relevance in nanoparticle-based therapies and in the development of Mφ cell therapies. Mφ play a role in maintaining immune homeostasis through processes like apoptotic body clearance, cytokine production, tissue remodelling, and the induction of regulatory and effector T-cell responses [18].

For these reasons, our aim was to gain a better understanding of the T1D-specific alterations that may hinder ASITs. First, we identified Mφ as the most impacted APC in non-obese diabetic (NOD) mice –the spontaneous model of T1D [19] – upon PS-liposome administration. We then characterised the phenotypic and functional features of this population in humans, before and after exposure to PS-liposomes. PS-liposomes were found to interact with four different subclasses of MoMφ from controls and individuals with T1D. Interestingly, we demonstrate that Mφ from T1D subjects displayed an altered expression of surface molecules. Besides, PS-liposomes induced changes in the phenotypic and cytokine release profiles of human Mφ, resulting in the down-modulation of T cell proliferation in the context of autoantigen stimulation.

Altogether, our results contribute to uncovering the alterations of myeloid cells in the context of T1D, a phenomenon not yet fully comprehended, and expand our understanding of the involvement of Mφ in the abrogation of autoimmune responses following ASITs.

2. Material and methods

2.1. Liposome manufacturing

Liposomes were manufactured using a solvent-injection method in a sterile environment at Ahead Therapeutics' cleanroom facility (l'Arboç, Catalonia, Spain). Liposomes formulation included 2-dioleoylsn-glycero3-phospho-L-serine or phosphatidylserine (Lipoid), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (Lipoid) and cholesterol (Sigma Aldrich). Lyophilised phosphatidylserine, phosphocholine and cholesterol were dissolved in ether at 55 °C and injected into a solution of sterile-filtered phosphate-buffered saline (PBS). For insulin-loaded liposomes, lyophilised insulin (Sigma Aldrich) was dissolved into PBS prior to lipid injection. Liposomes were extruded through a 1 μm pore membrane (Nucleopore Track-Etch Membranes, Whatman) with Lipex® Extruder (Evonik). After extrusion, the PS-liposomes were dialysed for 24 h and a milky homogeneous suspension was obtained. The critical quality attributes of the liposomal formulations, summarized in Table 1, were evaluated by Ahead Therapeutics. For the manufacture of fluorescent liposomes, 3-hexanoyl-Nitrobenzoxadiazole (NBD)-tagged cholesterol was used in the synthesis process.

2.2. Mice

NOD mice (obtained from Jackson Laboratory) were bred under specific-pathogen-free conditions at the Centre for Comparative Medicine and Bioimage (Badalona, Catalonia, Spain). All experiments were conducted following the guidelines of the Declaration of Helsinki for animal experimental investigations and the Principles of Laboratory Animal Care established by the National Institute of Health. The experimental protocols received approval from the Animal Ethics Committee at the Germans Trias i Pujol Research Institute and the Catalan Government (approval number DMA12139).

2.3. In vivo capture of fluorescent PS-liposomes in NOD mice

Insulin-loaded NBD-PS-liposomes (100 μL) were intravenously administered to prediabetic female NOD mice aged 8–10 weeks as previously described [8]. Mice were euthanized at 15 min and 6 h post-injection, and their spleens and livers were harvested. The spleen and liver were homogenized to obtain a single-cell suspension. For liver processing, the Liver Dissociation kit mouse (Milteny Biotec) was used following the manufacturer's instructions. Red blood cells were lysed using a haemolysis buffer containing Trizma® hydrochloride (Sigma-Aldrich) and NH_4Cl. Cells were stained for flow cytometry with CD45 APC/Fire810, CD19 BV570, CD14 PE-CF594, CD68 BV605 (BioLegend), CD3 PercP, CD11c BV786, B220 BV510, CD205 BV421, MHC-II PE and F4/80 APC-R700 (BD Biosciences). Fluorescence Minus One (FMO) staining was performed using male NOD mice and used to set the gates. All samples were processed using a FACS LSR Fortessa flow cytometer (BD Biosciences). Data was analysed using FlowJo software (BD Biosciences).

2.4. Subjects

Blood samples were obtained from adults with T1D (n = 16; 72% female, 28% male) and controls (n = 18; 56% female, 44 % male) at the Endocrinology Department of the Germans Trias i Pujol Hospital. All participants provided their informed consent before participation, and all procedures were conducted in strict accordance with approved guidelines. For individuals with T1D, the inclusion criteria ensured the selection of participants aged between 18 and 55, with a Body Mass Index (BMI) below 30, T1D duration of more than 6 months, absence of other autoimmune diseases or pregnancy, and no prior exposure to anti-inflammatory or immunosuppressive treatments. Ethics approval for all experimental protocols was granted by the Research Ethics Committee of the Germans Trias i Pujol University Hospital (approval number PI-22-297).

Clinical features and metabolic data from control subjects and patients with T1D are summarized in Table 2. Briefly, the age of control subjects was 28.25 ± 5.07 years (mean ± SD), while that of patients

Table 1
Critical quality attributes of PS-liposomes.

PS-liposome: physicochemical properties				
Liposome type	Fluorochrome	Diameter (nm)	Polydispersity index (PDI)	ζ-potential (mV)
PS-Liposome	–	474 ± 11 nm	0.34 ± 0.04	−24.6 ± 1,4 mV
PS-Liposome^{INS}	–	496 ± 7 nm	0.31 ± 0.04	−25.0 ± 0.9 mV
NBD-PS-Liposome^{INS}	NBD	512 ± 6 nm	0.30 ± 0.01	−24.3 ± 1.4 mV

Data presented as mean ± SD. INS, insulin; NBD, 3-hexanoyl-nitrobenzoxadiazole; PDI, polydispersity index.

Table 2
Data from participants with T1D and controls included in this study.

Parameter	Control subjects	T1D subjects
n	16	18
Sex (F/M)	9/7 (56%/44%)	13/5 (72%/28%)
Age (years)	28.25 ± 5.07	30.28 ± 6.42
BMI (kg/m^2)	23.5 ± 2.94	23.4 ± 2.65
Age at diagnosis (years)	NA	18.05 ± 7.94
Progression (years)	NA	12.94 ± 6.16
HbA1c (%)	NP	7.38 ± 1.08
Insulin dose (IU/kg/day)	NA	36.56 ± 12.94

Data presented as mean ± SD; p-value calculated from Mann-Whitney test. T1D, type 1 diabetes; BMI, body mass index; HbA1c, glycated haemoglobin; IU, insulin units; NA, not applicable; NP, not provided.

with T1D was 30.35 ± 7.15 years; BMI was 23.5 ± 2.94 kg/m2 and 23.4 ± 2.65 kg/m^2, respectively. No significant differences were observed in these parameters between the control and diabetic groups. Patients with T1D had been diagnosed at 18.05 ± 7.94 years of age, had a duration of disease of 12.94 ± 6.16 years, a glycated haemoglobin (HbA1c) level of 7.38 ± 1.08% and received an insulin dose of 36.56 ± 12.94 IU/kg/day. HbA1c and insulin dose were within the expected normal values for adults with T1D.

2.5. In vitro macrophage differentiation

Peripheral blood mononuclear cells (PBMCs) were obtained from participants using Ficoll Paque (GE Healthcare) density gradient centrifugation. CD14$^+$ cells were isolated using the EasySep™ Human CD14$^+$ Selection Kit II (STEMCELL Technologies™) as per the manufacturer's instructions. Viable CD14$^+$ cells were quantified using flow cytometry, with a 7-AAD viability stain (BD Biosciences) and an CD14 PE antibody (Immunotools). Fresh CD14$^+$ cells were seeded at a concentration of 4*10^5 cells/ml in X-vivo medium (Lonza), supplemented with 2% human AB serum (Biowest), 100 IU/ml penicillin (Normon SA), 100 μg/ml streptomycin (Laboratoris Reig Jofre), and 50 ng/ml of macrophage colony stimulating factor (M-CSF) (Prospecbio). The culture medium was renewed on day 3 and 6. For the obtention of different Mφ subtypes, polarising stimuli were added on day 6 and Mφ were collected on day 8. Stimulation protocols included for unpolarised (M0) MoMφ, or in combination with lipopolysaccharide (LPS) (100 ng/mL) (Sigma Aldrich) and interferon-γ (IFN-γ) (20 ng/ml) (Preprotech) for inflammatory (M1) MoMφ, IL-4 (80 ng/mL) (Prospecbio) for tissue-repair (M2a) MoMφ, and betamethasone or dexametasone (100 nM; 39.24 ng/mL) for deactivated (M2c) MoMφ. When mentioned, granulocyte-macrophage colony stimulating factor (GM-CSF) was used instead of M-CSF.

2.6. PS-liposome uptake assay

Following MoMφ complete differentiation, cells were incubated for 30 min, 1, 2, 4, and 24 h with 100 μM NBD-labelled insulin-encapsulating PS-liposomes, at 37 °C and 4 °C. The wells were thoroughly washed to remove any remaining liposomes, and MoMφ were detached using Accutase® (eBiosciences). Liposome uptake was then estimated by flow cytometry (FACS CantoTM II, BD Biosciences) as the percentage of NBD$^+$ MoMφ (CD14$^+$ CD11c$^+$). FMO staining for NBD was performed using non-fluorescent insulin-encapsulating PS-liposomes.

2.7. Monitoring of MoMφ viability

The Incucyte® live cell analysis system (Sartorius Stedim) was used to assess cell membrane integrity over time in different subclasses of MoMφ following PS-liposome co-culture. Briefly, MoMφ were cultured for six days with M-CSF, and the polarising stimuli were added along with propidium iodide (PI) and PS-liposomes. Sodium azide (NaN3) was used at 0.1% (weight/volume) as a positive control for cell death. Throughout the assay, single images per well, both phase contrast and fluorescence orange emission (excitation wavelength: 661 nm; passband: 546, 568 nm), were acquired with the SX5 G/O/NIR optical module at 10× magnification. One single image was captured every hour for 72 h, and each condition was assayed in duplicate. Automated real-time evaluation by live cell analysis included Propidium Iodide (PI) fluorescence intensity for all cells measured. Image analysis and data processing were performed with Incucyte® software version 2021C.

2.8. Confocal microscopy imaging

Monocytes were seeded in glass coverslips in 24-well plates (SPL Life Science Co.) and differentiated into MoMφ for 8 days. MoMφ were incubated for 24 h with 100 μM NBD-labelled insulin-encapsulating PS-liposomes. Next, wells were thoroughly washed to remove any remaining liposomes. Adherent cells were fixed with 10% formaldehyde, permeabilized in PBS +0.25% Triton X-100 and stained with phalloidin-Atto565 and DAPI (ThermoFischer). Dako fluorescent mounting medium (Sigma Aldrich) was then used to preserve the sample. Images were acquired on Zeiss Axio Observer Z1/LSM 710.

2.9. Macrophage immunophenotyping

MoMφ were treated with 1 mM PS-liposomes on day 6 and harvested on day 8 with Accutase® enzyme (eBiosciences) for subsequent downstream processing. Human MoMφ were stained with UV420 viability stain (Invitrogen), Human TruStain FcX™, True-Stain Monocyte Blocker™ (BioLegend), BD Horizon™ Brilliant Stain Buffer Plus (BD Bioscience), and 11 antibodies against surface markers: CD14 Spark Blue 550, CD36 BV421, CD200R PE/Dazzle 594, programmed death-ligand (PDL)-1 PE-Cy7 (Biolegend), CD80 PE (Immunotools), CD163 BV711, CD206 PE-Cy5, PDL-2 BV786 (BD Bioscience). All samples were processed using a Cytek Aurora™ spectral flow-cytometer (Cytek Biosciences). Data was analysed using SpectroFlo® software (Cytek Biosciences) and Flowjo™ software (BD Bioscience). To enable comparison between experiments, the phenotypic data of macrophages was normalised by dividing each data point by the mean of its respective experiment, employing the formula $X_{\text{normalised}} = \frac{X}{mean(X)}$.

2.10. Luminex® and enzyme-linked immunosorbent assay (ELISA)

Supernatants from MoMφ cultures (n ≥ 5 for controls and n ≥ 7 for individuals with T1D) were collected after complete differentiation in the presence or absence of PS-liposomes and frozen at −20 °C until use. Interleukin (IL)-1β, IL-12, IL-23, tumour necrosis factor alpha (TNFα), indoleamine-2,3-deoxygenase (IDO), Growth arrest-specific 6 (GAS6), vascular endothelial growth factor (VEGF)-A, and C–C motif chemokine ligand 2 (CCL2) were quantified using Luminex® multiplex kit. Transforming growth factor beta (TGF-β) was measured using Luminex® simplex kit (ThermoFischer Scientific) following the manufacturer's instructions. Samples were analysed using Luminex® 200. IL-6 and IL-10 were quantified using enzyme-linked immunosorbent assay (Fine Test).

2.11. Autologous T cell proliferation assays

Autologous T lymphocyte proliferation (n = 5) was assessed by exposing PBMCs from individuals with T1D to APCs, MoMφ, as previously described [7]. Briefly, cryopreserved CD14$^+$ cells were thawed and after confirming that viability was higher than 80%, cells were differentiated into M0, M1, M2a and M2c MoMφ. To induce autoreactive T cell proliferation, on day 6 human insulin (25 μg/ml) was added to the media together with the polarising stimuli. Insulin-loaded PS-liposomes were added 2 h afterwards. On day 8, autologous PBMCs were thawed

and after determining that viability was higher than 80%, cells were stained with 0.31 μM CellTrace™ Violet (ThermoFisher Scientific) according to the manufacturer's instructions. PBMCs were then co-cultured with MoMφ at a 1:5 ratio ($2*10^4$ MoMφ:10^5 PBMCs). In parallel, 10^5 PBMCs were cultured as a negative control in basal conditions and as a positive control with Phorbol 12-Myristate 13-Acetate (50 ng/ml, Sigma Aldrich) and Ionomycin (500 ng/ml, Sigma Aldrich). After 7 days of co-culture, $CD4^+$ and $CD8^+$ T cell proliferations were assessed with CD3-PE, CD4-APC and CD8-FITC staining (Immunotools) by flow cytometry (FACS LSR Fortessa™, BD Biosciences). Data was analysed using FlowJo software (BD Biosciences).

2.12. Statistical analysis

Statistical analysis was performed using the Prism 9.0 software (GraphPad Software Inc., San Diego, CA) and R-studio (RStudio, PBC, Boston, MA). The normality test was used to verify the normality of the data and the F-test to assess the SD of distinct populations when compared. For comparisons of unpaired data, a nonparametric Mann–Whitney test was used. For paired comparisons, a non-parametric Wilcoxon test was used. The analysis of variance (ANOVA) was used for comparisons with several factors, the Friedman test was used for the analysis of 3 or more paired groups and the Kruskal-Wallis when ANOVA assumptions were not met. A p-value <0.05 was considered significant.

3. Results

3.1. Murine splenic and hepatic Mφ are the most in vivo phagocytic cells of PS-liposomes

As a first step, we generated fluorescent PS-liposomes encapsulating human insulin (Table 1) hereafter referred to as NBD-PS-liposomes^{INS}. Intravenous (i.v.) administration of NBD-PS-liposomes^{INS} was performed in pre-diabetic female NOD mice to reach a total blood lipid concentration of 1 mM. Afterwards, the spleen and liver were harvested at 15 min and 6 h after administration. After processing the organs, splenic and hepatic immune cell populations were examined by flow cytometry and NBD fluorescence was assessed. T cells, B cells, DCs, Mφ and liver sinusoidal endothelial cells (LSECs) were characterised due to their role in orchestrating therapeutic responses to other preclinical ASITs.

NBD signal was detected in bulk cells of the liver and spleen at both time points. Fifteen minutes after the injection, 2.50% of $CD45^+$ cells were NBD^+ in the spleen and 28.83% in the liver. At 6 h, NBD positivity decreased to 1.61% and 15.27%, respectively (Fig. 1A). Regarding cell subsets (Fig. 1B), splenic and hepatic DCs reached high levels of fluorescence, being 15.14% and 33.51% at 15 min and 6.52% and 28.56% at 6 h, respectively. Remarkably, we identified Mφ as the most phagocytic cell subset. Mφ exhibited a 2-fold higher percentage of PS-liposome capture in the spleen (33%) compared to DCs at 15 min, and this disparity was even more pronounced in the liver, reaching a 3-fold difference (97.13%). In addition, while the percentage of fluorescent DCs significantly decreased after 6 h, Mφ maintained similar levels of NBD-PS-liposomes^{INS} capture at both time points.

This data underscores the significant role of the liver and spleen as primary sites for the uptake of PS-liposomes and ranks Mφ as the most phagocytic cell subset, highlighting their potential role in the processing of PS-liposomes.

3.2. The phagocytic function remains intact in MoMφ from patients with T1D

Having previously demonstrated a tolerogenic effect of PS-liposomes in human monocyte-derived dendritic cells (MoDCs) from patients with T1D [7], here we characterised the ability of human MoMφ to engage with PS-liposomes and capture them. We first confirmed the nature of the interaction by confocal microscopy. MoMφ were incubated with PS-liposomes (100 μM) and observed after 24 h. NBD-PS-liposomes^{INS} were detected within the cells' cytoplasm, confirming their internalisation (Fig. 2A).

Next, we conducted a time course capture experiment to determine the phagocytic ability of different MoMφ subtypes derived from controls or subjects with T1D. Precisely, unpolarised (M0-MoMφ), inflammatory (M1-MoMφ), tissue-repair (M2a-MoMφ) and deactivated (M2c-MoMφ) Mφ were incubated with NBD-PS-liposomes^{INS}. In a first step, we determined the limiting PS-liposome concentration to look for differences between subtypes. At 10 μM, 100 μM and 1 mM PS-liposome concentration, the percentage of NBD^+ MoMφ reached 15.29 ± 6.74, 64.18% ± 13.71 and 97.28% ± 1.45 after 24 h (Fig. S1A). Being the standard deviation at 100 μM the greatest before saturation, 100 μM PS-liposome concentration was selected for subsequent capture experiments (Fig. 2B).

As observed, there were no significant differences in the capture kinetics (Fig. 2C) nor area under the curve (AUC) (Fig. S1B) of M0, M1, M2a and M2c MoMφ between the control and T1D group at any given time point (30 min, 1 h, 2 h, 4 h, and 24 h). Additionally, the median fluorescence intensity (MFI) of NBD, which is indicative of the total number of internalised liposomes, was analysed at 24 h (Fig. 2D). Our results further demonstrated no differences between the T1D and control groups. As expected, NBD-PS-liposome^{INS} uptake was dramatically reduced at 4 °C indicating that liposome capture is a metabolically active process (Fig. S1C).

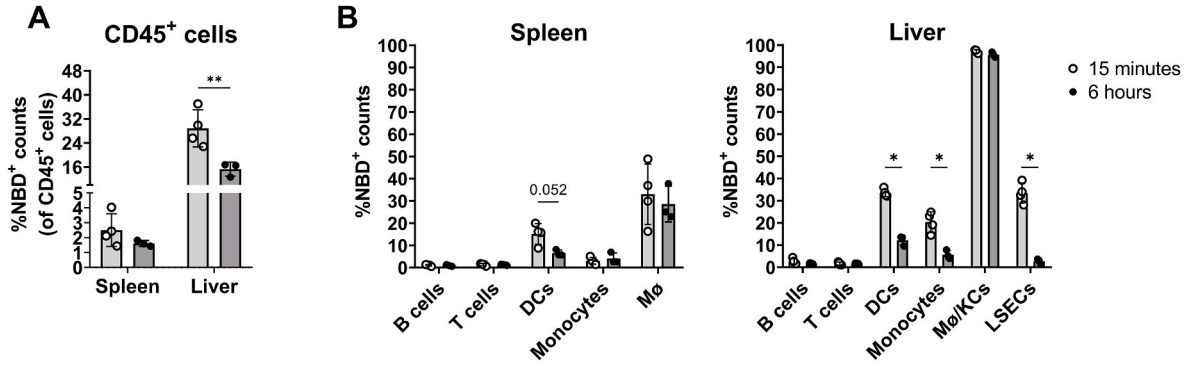

Fig. 1. Tracking fluorescent NBD-PS-liposomes in non-obese diabetic (NOD) mice. Percentages of NBD-fluorescent cells of the parent gate (A) in the spleen and liver (B) of NOD mice treated with NBD-PS-liposomes^{INS} intravenously, 15 min (n = 4) and 6 h (n = 3) after exposure. Cells were determined as follows: T cells, $CD45^+CD3^+$; B cells, $CD45^+CD19^+$; DCs, $CD45^+$, F4/80, $cD11c^{High}$, $MHCII^+$; Monocytes, $CD45^+$, $F4/80^-$, $CD11c^-$, $CD68^{Low}$, $CD14^+$; Macrophages (Mφ) and Kupffer cells (KC), $CD45^+$, $F4/80^+$, $CD68^+$, $CD11c^{Low}$; Liver sinusoidal endothelial cells (LSEC), $CD45^-$, $CD206^+$. Data is displayed as mean ± SD. Differences were found when comparing time points within the same subset (ns ≥ 0.05, *p < 0.05, **p < 0.01, two-way ANOVA with Tukey's multiple comparison test).

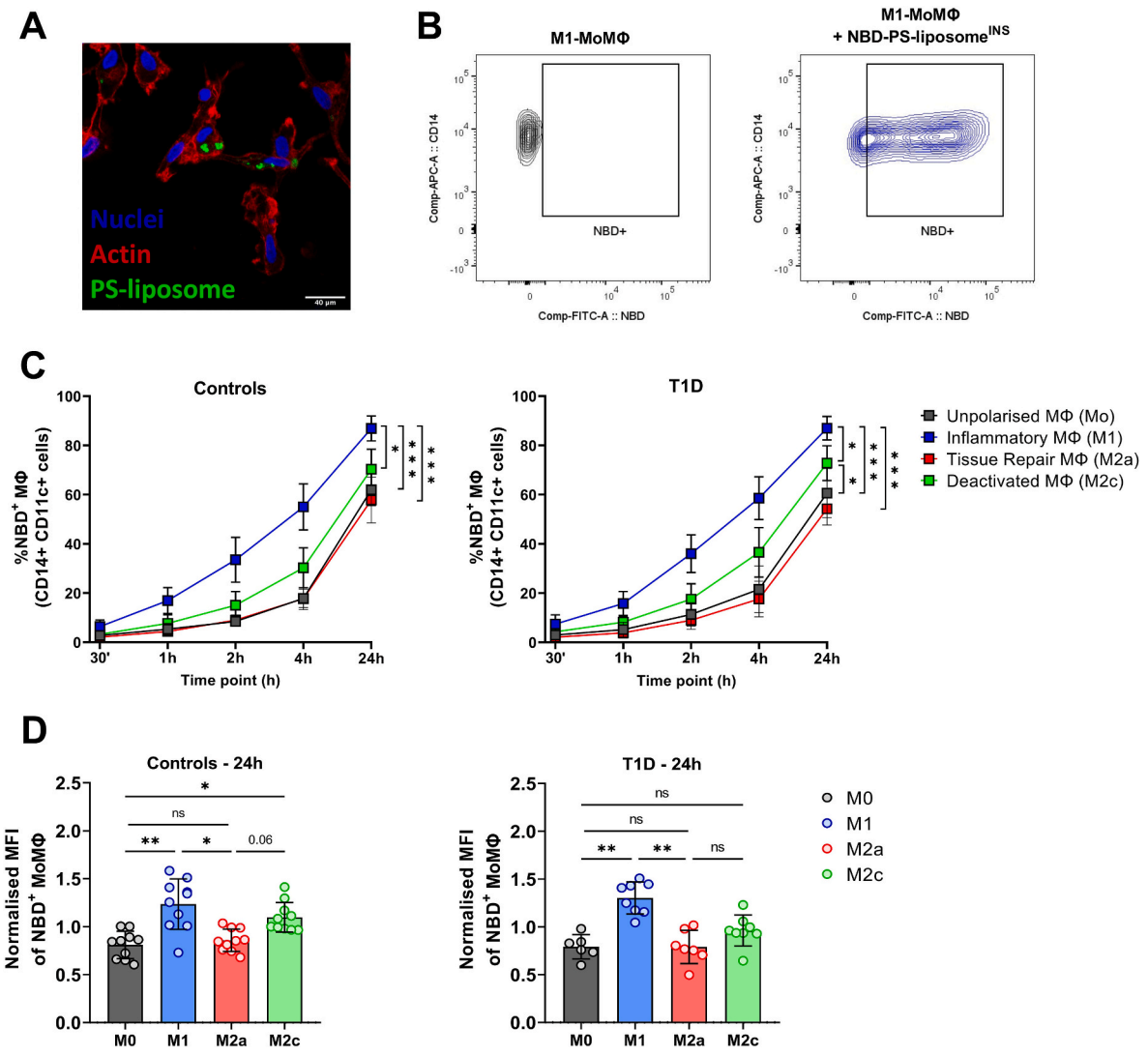

Fig. 2. The phagocytic ability remains intact in Mφ from patients with T1D. (A) Representative confocal microscopy imaging of inflammatory M1 macrophages after 24 h of coculture with NBD-PS-liposomes^{INS}. PS-liposomes in green, phalloidin in red and DAPI in blue. (B) Representative dot plot illustration of M1-MoMφ after 24 h incubation with 100 μM non-fluorescent (left) and NBD-fluorescent (right) PS-liposomes, data analysed by flow cytometry. (C) Time-course experiment displaying the dynamics of NBD-PS-liposomes^{INS} capture by MoMφ derived from control subjects (n = 10), and subjects with established disease (n = 8) at 37 °C and 100 μM concentration. (ns ≥ 0.05, *p < 0.05, **p < 0.01, Mixed one-way ANOVA) (D) Relative MFI of NBD^{+} MoMφ after 24 h of coculture with NBD-PS-liposomes^{INS}. Data displayed as mean ± SD. (ns ≥ 0.05, *p < 0.05, **p < 0.01, Friedman and Kruskal-Wallis tests with Dunn's multiple comparison test).

On the other hand, significant differences were discernible between the capture ability of the four distinct MoMφ subtypes in terms of capture kinetics (Fig. 2C), MFI (Fig. 2D) and AUC (Fig. S1B). Inflammatory M1-MoMφ ranked as the most phagocytic, followed by the deactivated M2c-MoMφ, and then by the tissue-repair and unpolarised MoMφ whose rates of NBD-PS-liposome^{INS} uptake did not differ significantly.

These results demonstrate that the ability to *in vitro* phagocytosis of PS-liposomes by MoMφ from individuals with T1D is not impaired. This supports the effectiveness of PS-liposomes as tolerogenic antigen-delivery vehicles. Although previous studies have reported impaired phagocytosis of bacterial particles in individuals with hyperglycaemia and type 2 diabetes [20], our results, using PS-liposomes and pure MoMφ cultures, discard any impairment in the phagocytic activity of Mφ in the context of T1D.

3.3. PS-liposome internalisation does not impact MoMφ viability

To ensure that the engulfment of PS-liposomes did not compromise cell viability, we monitored the effect of saturating PS-liposome concentrations on MoMφ in real time with the Incucyte® live cell analysis system. M0, M1, M2a and M2c MoMφ were monitored for 72 h, and cell death was quantified with PI nuclear stain. We identified no significant effect of 100 μM and 1 mM PS-liposome concentrations in the viability of M0, M1, M2a and M2c MoMφ from subjects with T1D (Fig. 3) and controls (Fig. S2) when compared to not treated MoMφ. In contrast, the presence of 0.1% NaN_3, a well-characterised metabolic inhibitor, triggered progressive cytotoxicity in MoMφ as observed by the increase in PI intensity (Fig. 3).

These findings fit well with previous experiments in DCs [7] and confirm the innocuity of PS-liposomes *in vitro* in different Mφ subclasses encouraging further development. Critically, this is a cornerstone parameter that has excluded certain nanoparticles and liposomal formulations from clinical development [21,22].

3.4. MoMφ expression of phenotypic markers is altered in T1D subjects

Individuals with T1D often encounter unstable levels of blood glucose and inflammatory mediators, which can influence the

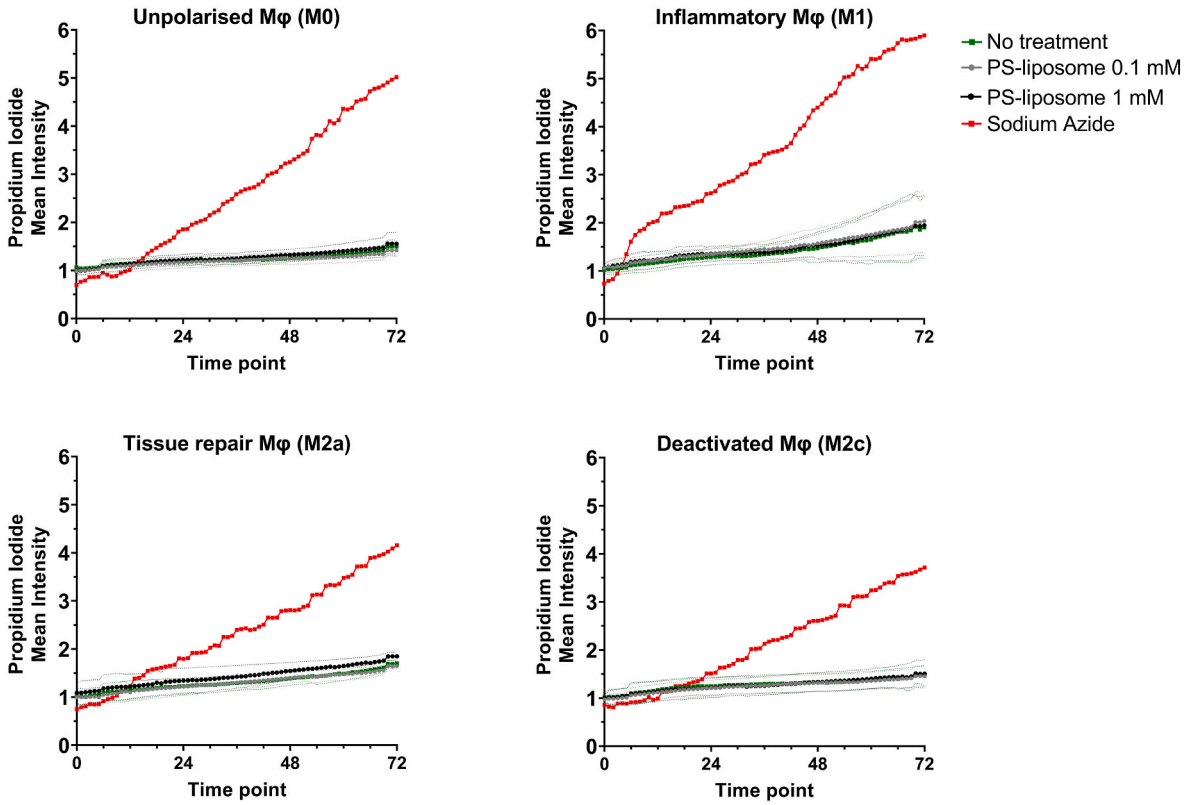

Fig. 3. Effect of PS-liposomes on the viability of MoMφ from T1D subjects. Incucyte® cell imaging system was used to assess the effect of 100 μM and 1 mM PS-liposomes on the viability of monocyte-derived macrophages obtained from subjects with T1D (n = 3). Propidium Iodide (PI) was used as a marker for the loss of membrane integrity and NaN_3 (0.1%) as a positive control. Figures display a time course of the orange mean fluorescence intensity (MFI) of the cells throughout 72 h of observation.

homeostasis of the immune system [23]. This includes alterations in its tolerogenic potential, as previously reported in APCs [24,25]. Here we determined the immune-related phenotype and function in four subclasses of MoMφ from subjects with T1D ($n \geq 12$) and compared it to controls ($n \geq 11$). Specifically, molecules related to antigen presentation (CD80, CD86, HLA-DR, PDL-1 and PDL-2), PS binding (CD14, CD36 and MER proto-oncogene, tyrosine kinase (MERTK)) and anti-inflammatory Mφ-polarisation (CD163, CD200R and CD206), were selected. The relative expression of molecules for each condition is shown in Fig. 4. The polarisation of M2c-MoMφ with betamethasone and dexamethasone was examined, resulting in a comparable expression of phenotypic markers in both T1D subjects and controls (Fig. S3). Consequently, betamethasone was chosen for subsequent experiments.

The expression patterns of the four MoMφ subtypes within the control and T1D groups were similar. Specifically, CD80, CD86, PDL-1, PDL-2 and CD14 expression was prominent in M1-MoMφ, while CD200R and CD206 were notable in M2a-MoMφ. M2c-MoMφ exhibited high expression of CD80, CD163 and MERTK. Additionally, low expression of PDL-1 was a characteristic feature of the deactivated MoMφ group. In contrast, M0-MoMφ did not display distinctive expression of any studied markers, with low and homogeneous expression being its most characteristic phenotypic trait (Fig. 4A).

After correction for multiple comparisons, the comparative analysis of the data between controls and T1D subjects revealed subtle differences in various Mφ subsets (Mo, M2a, and M2c, as illustrated in Fig. 4B). Notably, individuals with T1D showed reduced CD36 expression in M0-MoMφ ($p = 0.16$). In M1-MoMφ, the expression of CD80 tended to be lower in the T1D group ($p = 0.09$), and remarkable, for this subtype, four individuals from the T1D group lost the expression of CD86 and HLA-DR (see Section 3.5). M2a-MoMφ of the T1D group displayed heightened HLA-DR expression ($p = 0.01$), and phenotypic alterations also extended to the M2c subtype, in which we observed a tendency to increase CD80 ($p = 0.05$) and lowered CD163 levels ($p = 0.12$). Most of the molecules studied remained within comparable levels of expression, suggesting an overall similar behaviour in the response of both groups to monocyte-polarising stimuli.

In summary, these data suggest that M0, M2a and M2c MoMφ *in vitro* differentiated do not suffer from relevant impairments in the context of T1D despite nuanced phenotypic alterations in certain markers. However, it also points out great impairments in the differentiation of M1-MoMφ in a subgroup of individuals with T1D.

3.5. *Aberrant response to inflammatory stimuli in M–CSF–driven MoMφ is a T1D oddity*

Surprisingly, we identified certain individuals with T1D who exhibited an atypical phenotypic outcome to M1-MoMφ polarisation with inflammatory stimuli. Four out of the fourteen T1D participants included in this experiment (28%) lost the expression of CD86 and HLA-DR, either partially or entirely, hereafter referred to as "non-responders" (Fig. 4A and B). Intriguingly, this loss of HLA-DR correlated with a pronounced increase in CD163 expression ($r = -0.824$) (Fig. S4A) and a high CD80/CD86 ratio (Fig. S4B), resulting in an aberrant M1-MoMφ phenotype closely resembling that of deactivated M2c-MoMφ.

To confirm these findings and rule out potential artefactual cytometry events, we determined the loss of CD86 and HLA-DR in non-responders after a second blood withdrawal and conventional flow cytometry analysis (FACS Canto II, BD Biosciences) with similar results (data not shown). Significantly, none of the subjects in the control group exhibited a similar loss of CD86 and HLA-DR in inflammatory M1-MoMφ, strongly indicating the T1D-specific nature of this phenotypic behaviour. Additionally, other MoMφ subtypes (e.g., M0, M2a and M2c)

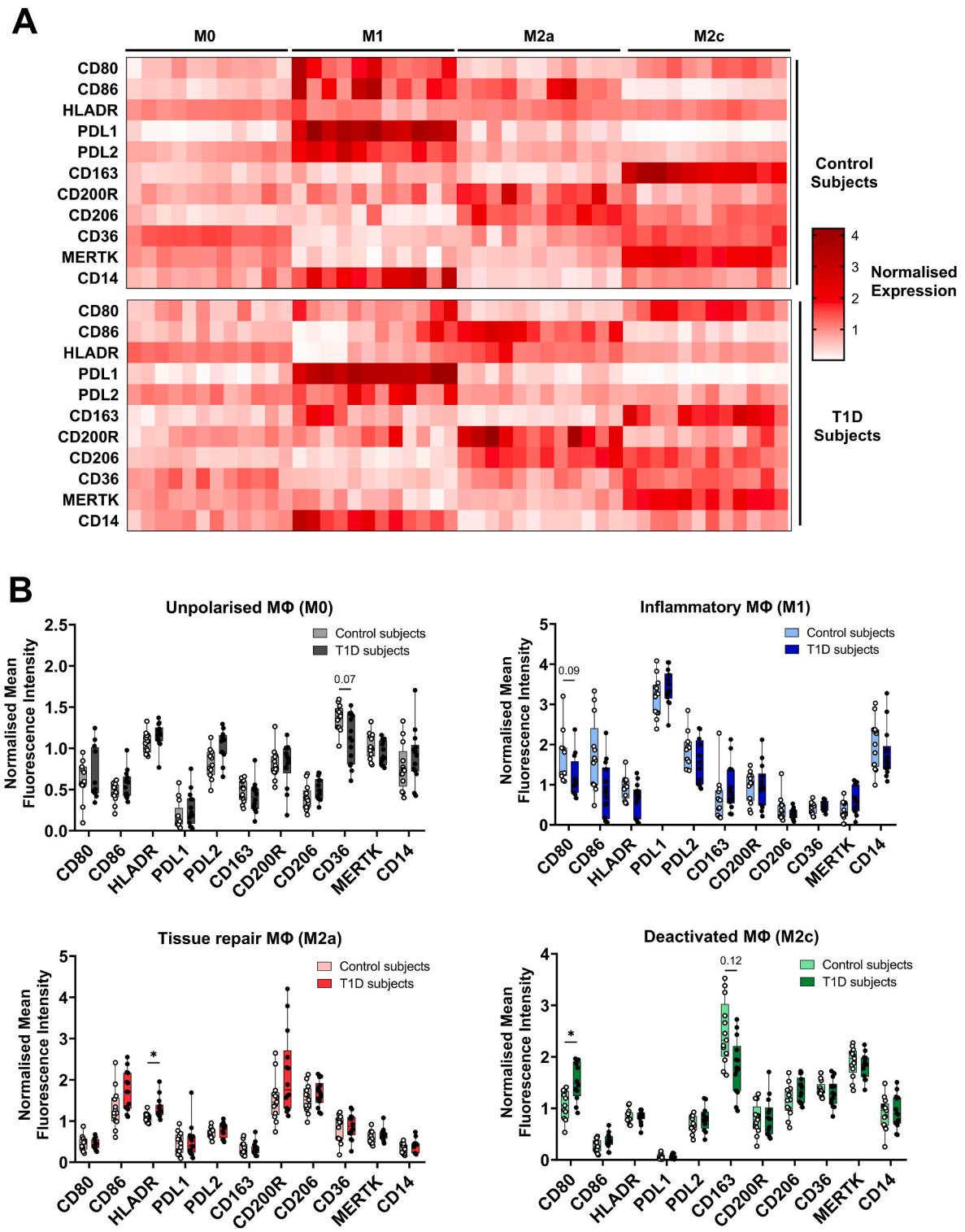

Fig. 4. Phenotypic characterisation of surface markers in MoMφ from T1D and control subjects. (A) Heatmap representing the expression of surface markers. Expression data from M0, M1, M2a and M2c monocyte-derived macrophages (MoMφ) from T1D (n = 12) and control (n = 11) subjects. (B) Normalised expression of CD80, CD86, HLA-DR, PDL-1, PDL-2, CD163, CD200R, CD206, CD36, MERTK, CD14 of M0, M1, M2a and M2c MoMφ obtained from T1D (n ≥ 12) and control (n ≥ 11) subjects. (ns ≥ 0.05, *p < 0.05, Mann-Whitney test with Holm-Sidák multiple's comparisons correction).

within the non-responder group showed normal levels of CD86, HLA-DR and CD163 when compared to controls, suggesting that the anomalous loss of these molecules is specific to inflammatory stimulation with LPS and IFN-γ.

Delving into this discovery, we tested other stimuli to differentiate and polarise M1-MoMφ in non-responder subjects. In contrast to M1-MoMφ differentiated with M-CSF and polarised with LPS and IFN-γ, GM-CSF driven differentiation was able to rescue the expression of CD86 and HLA-DR to the same levels as the responder and control groups (Fig. S5). Hence, this implies that pre-polarisation of non-responder monocytes with M-CSF prompts an aberrant response to LPS and IFN-γ.

These findings reveal a previously unreported T1D-specific

alteration, highlighting the particularities of monocyte to Mφ differentiation in the context of T1D. This underscores the importance of additional considerations in the development of new therapeutic strategies targeting Mφ.

3.6. PS-liposomes selectively modulate the immunoregulatory phenotype of non-inflammatory Mφ

Building upon our findings, we established that Mφ are the primary cell type responsible for the uptake of PS-liposomes *in vivo* in NOD mice and that MoMφ from subjects with T1D and controls demonstrated similar *in vitro* phagocytosis of PS-liposomes. Then, the phenotypic

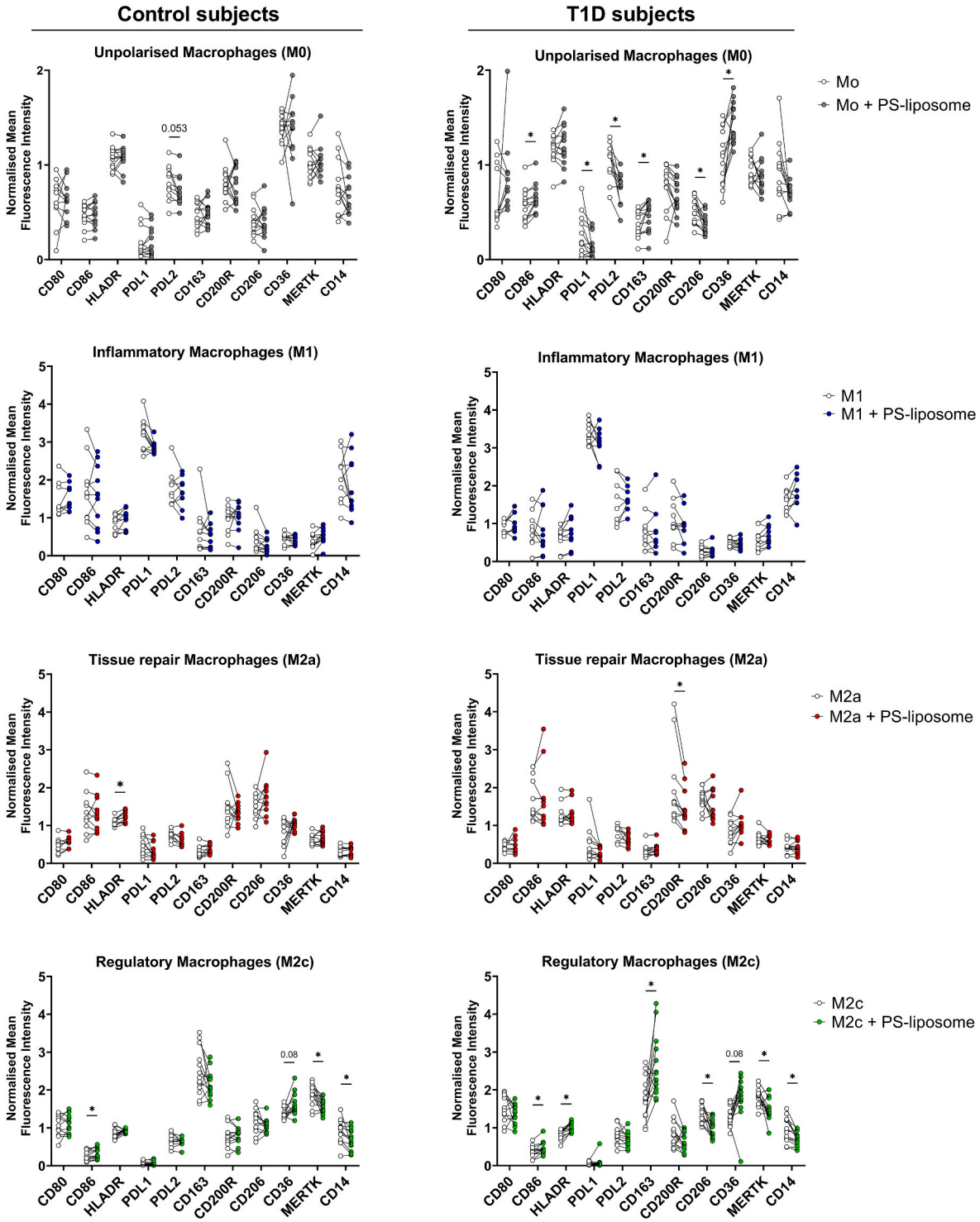

Fig. 5. PS-liposome capture by MoMφ finely regulates their phenotype. Normalised expression of CD80, CD86, HLA-DR, PDL-1, PDL-2, CD163, CD200R, CD206, CD36, MERTK, CD14 of M0, M1, M2a and M2c monocyte-derived macrophages (MoMφ) obtained from T1D ($n \geq 12$) and control ($n \geq 11$) subjects. Within each group, dots represent MoMφ (white dots) and MoMφ after 48 h of incubation with PS-liposomes (coloured dots). (ns ≥ 0.05, *$p < 0.05$, Wilcoxon test with Holm-Sidák multiple's comparisons correction).

changes induced by PS-liposome engulfment were determined. We studied the effect of PS-liposomes on monocyte polarisation by co-delivering the subtype-specific stimuli together with a saturating concentration of PS-liposomes.

Fig. 5 shows the effect of PS-liposomes on the phenotype of MoMφ. Regarding PS binding molecules, the expression of CD36 on M0-MoMφ, while marginally lower in the T1D group when compared to control group ($p = 0.12$, Fig. 4B), showed a significant increase after co-culture with PS-liposomes ($p = 0.02$) reaching levels comparable to those of the control group. In M2c-MoMφ, cells specialized in efferocytosis, PS-liposomes induced consistent changes in MERTK, CD36 and CD14 expression in T1D and control groups, reflecting the use of these receptors for internalisation [26–28]. On the other hand, the expression of PS binding molecules was preserved within the M1-MoMφ and M2a-MoMφ.

Interestingly, this intervention did not induce a phenotypic shift in the antigen-presenting molecules profile of M1-MoMφ. However, discernible effects were observed in the non-inflammatory Mφ (M0, M2a, and M2c). In M0-MoMφ, PS-liposome capture led to a uniform decrease in the levels of PDL-1 ($p = 0.04$) and PDL-2 ($p = 0.02$) checkpoint inhibitors in the T1D group and the same tendency was observed in controls for PDL-2. Simultaneously, a modest increase in CD86 levels was noted after PS-liposome uptake in the T1D group ($p = 0.01$). In M2a-MoMφ, the expression of PDL-1, PDL-2, CD80, CD86, and HLA-DR remained unchanged in the T1D group, while HLA-DR experienced a slight increase in the control group following PS-liposome therapy ($p = 0.02$). Similar changes were observed in M2c-MoMφ, where PS-liposome interaction led to an increase in the levels of CD86 and HLA-DR.

M2 polarisation markers also varied in M0, M2a, and M2c MoMφ, with the most notable variations observed in non-inflammatory MoMφ. In individuals with T1D, M0-MoMφ, CD163 expression increased after PS-liposome capture ($p = 0.04$), whereas CD206 expression decreased ($p = 0.02$). In contrast, CD200R membrane levels did not vary, mirroring the findings in the control group for CD163, CD206 and CD200R. Regarding the M2a-MoMφ, controls displayed no discernible phenotypic response to PS-liposome interaction. However, in subjects with T1D, PS-liposomes facilitated a phenotypic rescue, restoring CD200R expression levels to match those of the control group ($p = 0.02$). A similar PS-liposome-mediated phenotypic rescue was evident in M2c-MoMφ, where the low levels of CD163 in T1D MoMφ ($p = 0.12$) significantly increased ($p = 0.04$) to align with the expression levels of the control group. This trend was also observed for CD206, with slightly decreased levels after PS-liposome capture ($p = 0.02$).

These results point to a primary phenotypic effect of PS-liposomes on the non-inflammatory M0, M2a and M2c MoMφ subtypes, with minimal impact on the LPS + IFN-γ polarised M1-MoMφ.

3.7. *PS-liposome uptake selectively impacts the cytokine profile of inflammatory M1-MoMφ towards tolerance*

To further dissect the effect of PS-liposome therapy in Mφ function, we analysed the cytokine release upon their engulfment using Luminex and ELISA.

Cytokine release was predominantly low, often below the threshold for detection, in non-inflammatory M0-MoMφ and M2c-MoMφ. Noteworthily, we did not observe discernible differences between cytokine concentration levels in cell cultures from subjects with T1D and controls (Fig. S6). In contrast, M1-MoMφ had a remarkably higher secretion of inflammatory and regulatory cytokines (Fig. 6), presumably attributed to the potent differentiation stimuli (e.g. LPS and IFN-γ). Within M1-MoMφ, growth arrest-specific protein 6 (GAS6) secretion was lower in

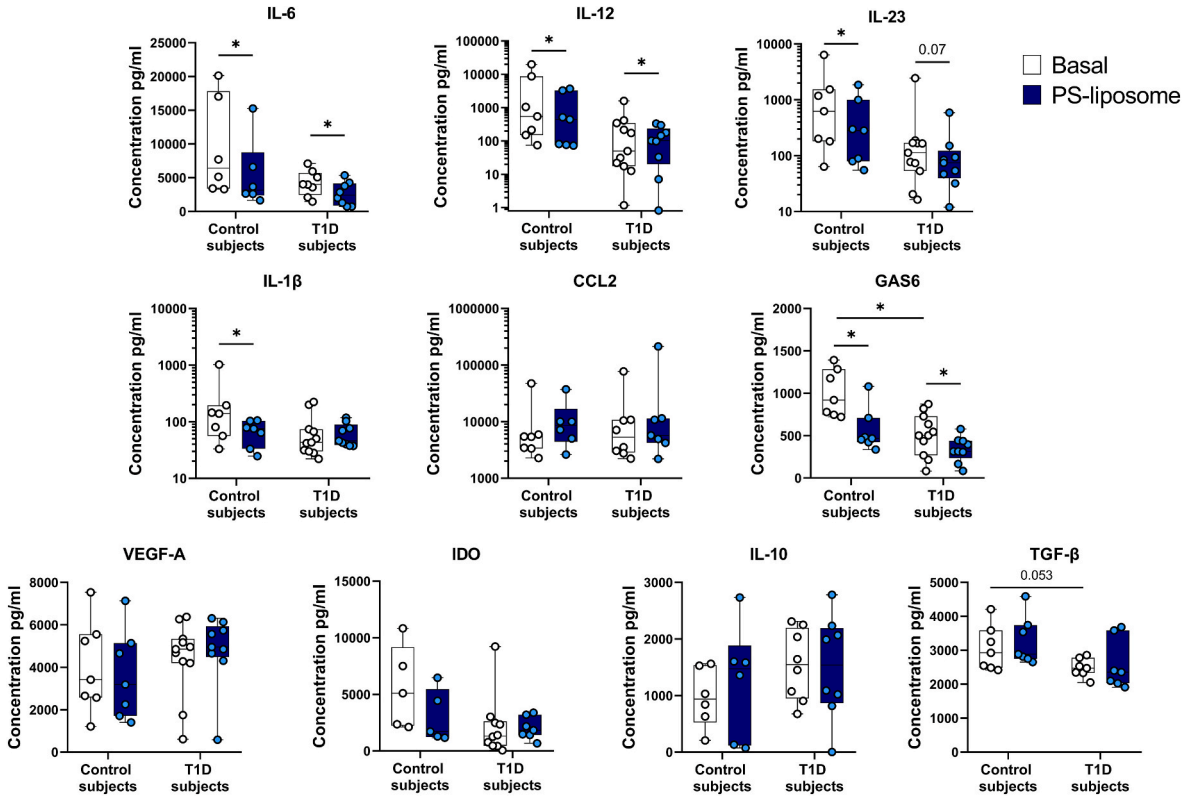

Fig. 6. Functional effect of PS-liposomes on the cytokine profile of inflammatory (M1) MoMφ. Levels of IL-6, IL-12, IL-23, IL-1β, CCL2, GAS6, VEGF-A, IDO, IL-10 and TGF-β secreted by inflammatory monocyte-derived macrophages (MoMφ) obtained from control subjects ($n \geq 5$), and patients with T1D ($n \geq 7$). Within each group, bars represent M1-MoMφ (white bars) and M1-MoMφ after 48 h of incubation with PS-liposomes (blue bars). Data is presented as mean ± SD. Significant differences were found when comparing the different conditions in the same group of subjects (ns ≥ 0.05, *p < 0.05, Wilcoxon test) or when comparing conditions between groups (ns ≥ 0.05, *p < 0.05, Mann-Whitney test).

the T1D group, independently of their classification as responders or non-responders and TGF-β displayed the same tendency ($p = 0.053$) (Fig. 6).

PS-liposome exposure of M0 and M2c MoMφ did not induce any significant changes, and preserved VEGF-A, IL-10, TGF-β and IDO secretion levels. In M1-MoMφ the tolerogenic effect of PS-liposome capture was particularly pronounced. PS-liposome-treated MoMφ significantly reduced the secretion of IL-6 and IL-12 in the T1D group and IL-1β, IL-6, IL-12 and IL-23 in Mφ from the control group, when compared to their non-treated counterparts (Fig. 6). Importantly, the secretion of other soluble mediators including IL-10, IDO, TGF-β and VEGF-A remained unchanged. Furthermore, the consistent decrease of GAS6 after liposome co-culture, indicates the efferocytic nature of PS-liposome internalisation and justifies the observed decrease in the release of inflammatory mediators.

Overall, these results demonstrate the effects of PS-liposome exposure on M1-MoMφ function. The targeted modulation of inflammatory Mφ subtypes, sparing key regulatory and tissue repair mediators like IL-10, IDO, TGF-β and VEGF-A highlights the potential therapeutic relevance of PS-liposome interventions in the context of inflammation.

3.8. *PS-liposome^{INS} exposure of inflammatory M1-MoMφ modulates CD4$^+$ and CD8$^+$ T cell responses in the context of autoantigen stimulation*

We previously demonstrated that PS-liposome capture impairs human immature DCs' ability to stimulate CD8$^+$ autologous T cell proliferation in the context of T1D [7]. Therefore, we aimed to investigate whether PS-liposomes would also induce a comparable tolerogenic effect in MoMφ. Our experimental approach involved co-culturing autoantigen-loaded MoMφ (i.e., human insulin) with autologous PBMCs from individuals with T1D. Notably, only the M1-MoMφ subclass was able to trigger autoreactive T cell proliferation (Fig. 7), which is in agreement with the heightened cytokine release of this group (Fig. 6). Autologous T cell proliferation stimulated by M0, M2a and M2c MoMφ was not observed. Furthermore, we detected that PS-liposome^{INS} phagocytosis impaired the M1 Mφ's capacity to induce autologous CD4$^+$ and CD8$^+$ T cell proliferation. This observation underscores the pivotal role of inflammatory Mφ in orchestrating T-cell responses in autoimmune disease, particularly within the context of autoantigen stimulation.

Altogether, the selective impact of insulin-loaded PS-liposomes on the M1 subtype suggests a specific functional vulnerability of M1 Mφ in which PS-liposomes may exert tolerogenic effects.

4. Discussion

It is well established that Mφ are essential phagocytes with crucial roles in the clearance of apoptotic cells and the maintenance of tolerance to prevent autoimmunity [18,29,30]. While several articles have reported the alterations of tolerogenic DCs in T1D [11,14,31–33], fewer studies have focused on human Mφ and their relation to self-tolerance recovery in autoimmune diabetes. Previous research indicates an altered monocytic stage in subjects with T1D, which does not seem to affect the functionality and phenotype of MoDCs [7]. However, the impact on MoMφ had not been explored. Experiments in mice suggest that a deficient immunomodulatory function in islet-resident macrophages could be a mechanism of pathogenesis in T1D [34]. Instead of silently clearing apoptotic cells, these cells secrete high amounts of pro-inflammatory cytokines [35]. To the best of our knowledge, this is the first study to assess the function of different human Mφ subsets and their potential in restoring self-tolerance in the context of T1D. Here, we identified Mφ as the primary capturers of tolerogenic nanoparticles *in vivo*. Subsequently, we characterised MoMφ in terms of phenotype, cytokine release and functionality upon co-culture with PS-liposomes in the context of autoimmune diabetes.

Our results underline the phagocytic ability of Mφ from NOD mice which is higher than that of other APCs. As previously reported [36] [–] [38], hepatic and splenic Mφ have been associated with the phagocytic clearance of apoptotic bodies and the promotion of self-tolerance. Indeed, several ASITs undergoing preclinical and clinical development focus on the delivery of disease-specific autoantigens to phagocytes of the human spleen and liver or to blood monocytes which are later recruited to the spleen to downmodulate autoreactive responses [39,40]. Further, cell transfer of immunosuppressive M2 Mφ to experimental models of T1D prevent the disease [41]. In light of these results, Mφ arises as a promising candidate for the induction of antigen-specific tolerance.

Focusing on translationality, we next characterised phagocytosis dynamics of human MoMφ against antigen-loaded PS-liposomes. Previous studies describe that glycaemic control correlates with the phagocytic activity of bacteria by blood phagocytes in the context of type 2 diabetes [20]. However, our results show that four subsets of human MoMφ from adult T1D individuals have an optimal capacity to engulf PS-liposome particles. This disparity might be due to the distinct nature of the particles employed in the respective studies and to the dissimilarities in the cell types and methods. Furthermore, the available data on MoDCs [7] from T1D donors aligns with our results, indicating no impairment in the ability of immune phagocytes to engulf

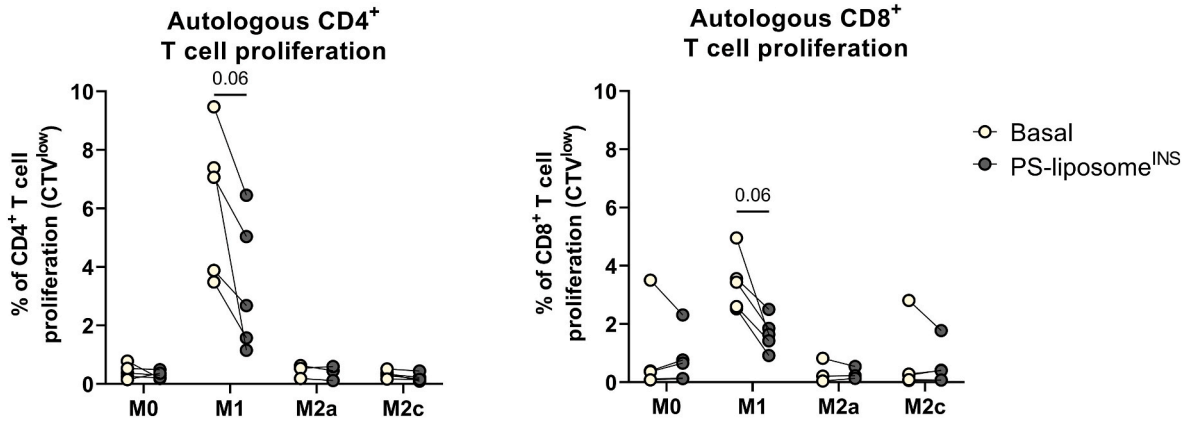

Fig. 7. Impact of PS-liposomes on autologous T cell proliferation. Percentage of CD4$^+$ and CD8$^+$ T cell subsets proliferation induced by autologous MoMφ in the presence of autoantigen (human insulin). Peripheral blood mononuclear cells (PBMCs) obtained from patients with T1D (n = 5) were stained with CellTrace Violet (CTV) and co-cultured at 1:5 ratio with autologous monocyte-derived macrophages (MoMφ) for 6 days. MoMφ were left untreated or treated with PS-liposomes^{INS} for 48 h before T cell co-culture. CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cell proliferation was measured as the percentage of CTV^{low} cells. Significant differences were found when comparing culture conditions within the same subtype of MoMφ (ns ≥ 0.05, Wilcoxon test).

PS-liposomes. Thus, PS-liposomes appear to be excellent candidates for the delivery of antigens in this therapy since their engulfment is not hindered by the progression of T1D. Interestingly, we observed dissimilar rates of PS-liposome capture among the four subtypes of Mφ studied which may be indicative of the Mφ subclasses that are targeted *in vivo* by PS-liposomes. Inflammatory MoMφ displayed the highest MFI and percentage of capture throughout the time course, indicating a proclivity of PS-liposomes to engage primarily with M1 activated Mφ. Little data on the phagocytic function of different human Mφ subsets is available. However, our findings replicate the results obtained by Dransfield et al. [26] which show a higher uptake of apoptotic bodies by LPS polarised Mφ. In contrast to that, authors employing particles of different nature (i.e., *E. coli*) report a distinct behaviour in M-CSF, IL-4, glucocorticoid and LPS + IFN-γ treated Mφ [42]. Of note, in our study, we used a non-disruptive method to quantify PS-liposome phagocytosis in contrast to the conventional phagocytosis protocols that involve harsh and cell-damaging processes such as enzymatic cell detachment [43]. Here we incubated PS-liposomes with untouched monolayers of MoMφ maximising cell viability along the experiment and a higher resemblance to the biological context *in vivo*.

Then, to discard substantial alterations in Mφ from T1D subjects, we dissected the phenotype of human Mφ from T1D and control subjects with special interest in molecules involved in phagocytosis and tolerance. We hoped to address if, prior to PS-liposome uptake, Mφ from patients and controls had similar characteristics. Unpolarised macrophages displayed equal characteristics in both groups and had a dim expression of most surface markers in comparison to other subtypes. Particularly, we observed nuanced differences between non-inflammatory Mφ from control and T1D individuals after polarisation with IL-4 and betamethasone. Although not profoundly impaired, CD80 and HLA-DR levels were increased in deactivated and tissue repair MoMφ, respectively, in the T1D group. It has been observed that higher CD80 to CD86 ratios are linked to a pronounced stimulation of T regulatory cells [44] and the induction of immune tolerance by DCs [45] due to the higher affinity of CD80 to the CTLA4 receptor expressed on regulatory cells [46]. Therefore, these changes reflect an attempt for immunoregulation in patients with autoimmunity which in turn also have higher levels of T regulatory cells in peripheral blood [47]. Furthermore, we also observed significant alterations in the phenotype of inflammatory M1-MoMφ that only affected a small group of the T1D participants included in this study, referred to as non-responders. Surprisingly, the aberrant loss of CD86 and HLA-DR in non-responders did not affect the other parameters studied since the cytokine release of the non-responder patients and their phagocytic ability ranked within normal values. These findings establish that monocytes from specific subjects with T1D suffer certain abnormalities that could impair their polarisation towards an inflammatory Mφ state if pretreated with M-CSF. Since M-CSF alone did not render aberrant Mφ and GM–CSF–driven polarisation of the inflammatory subtype, we hypothesise that the underlying cause of the observed phenomena relies on the malfunction of specific LPS or IFN-γ signalling pathways which are skewed after M-CSF exposure. In fact, dysregulation of the IRF4/IRF5 transcription factor axis, involved in the response to IFN-γ and LPS, has been reported in T1D [48] as well functional and metabolic responses in T1D monocytes upon stimulation of the toll-like receptor 4 (TLR-4) [33,49,50].

Next, we analysed the effect of PS-liposome capture on MoMφ. Despite the subtle differences in M0, M2a and M2c MoMφ between patients and controls, the response to PS-liposomes pointed to homeostasis recovery in the context of T1D and was most consistent in deactivated MoMφ. In the T1D group, PS-liposome uptake modified the expression of CD36 in M0, CD200R in M2a and CD163 in M2c MoMφ to match the levels of expression of the control group. Additionally, M0 showed an upregulation in CD36, CD86, and CD163 expression, and a decrease in PDL-1 and PDL-2 levels, resulting in a phenotype closer to the deactivated Mφ. On the other hand, deactivated macrophages exhibited a decrease in MERTK and CD14, suggesting the use of these receptors for the internalisation of PS-containing liposomes. This process may contribute to positive feedback loop, facilitating further liposome internalisation. Remarkably, PS-liposomes did not trigger any significant changes in the phenotype of the inflammatory M1-MoMφ. In this condition in which LPS and IFN-γ are present during PS-liposome exposure, the potential smooth homeostatic effect of PS-liposomes may not have been sufficient to counteract the potent inflammatory signal, resulting in a negligible impact in the phenotype. Although other studies have reported profound changes in inflammatory macrophages upon interaction with apoptotic bodies, the absence of other apoptotic determinants in PS-liposomes [51] may explain the subtle tolerogenic signal perceived in our experiments.

Nanoparticle-based ASITs confront many challenges in the pursuit of therapeutic efficacy. One obstacle is the heterogeneity of patients with T1D, including the distinct endotypes that affect the inflammatory state [52]. Ideally, nanoparticle-based therapies for autoimmunity should halt autoreactive responses by delivering disease-specific autoantigens to APCs. Nonetheless, were the targeted APCs are inflammatory, the therapy could face failure and worsen the evolution of the disease. Hence, antigen carrier nanoparticles ought to enforce a tolerogenic shift in these cells, particularly if these are not inherently tolerogenic. In the present study, we did not observe phenotypic changes in inflammatory MoMφ. Despite that, we demonstrate that while preserving the levels of VEGF-A, IL-10, IDO and TGF-β, PS-liposome phagocytosis reduced the secretion of pro-inflammatory cytokines in M1-MoMφ. This occurrence, together with a significant decrease in GAS6 levels after PS-liposome engulfment, highlights the involvement of efferocytic pathways in the response to PS-liposomes. Central to our discovery is that GAS6, a PS-binding opsonin that mediates the tolerogenic processing of apoptotic bodies, was highest in M1-MoMφ supernatants. PS-liposome opsonisation by GAS6 and subsequent efferocytosis by M1-MoMφ would explain the high rates of PS-liposome capture and justify in part the mechanism of action of PS-liposomes in this Mφ subtype. Aligning with our findings, a regression in the M1 Mφ phenotype to M2 upon exposure to PS has previously been reported [53]. It is worth mentioning that, in tumour-bearing mice, antibody-mediated blockade of PS signalling led to an induction of M1 polarisation further suggesting that PS can dampen the inflammatory state of Mφ [54]. In agreement with this data, a reduction in autologous $CD4^+$ and $CD8^+$ T cell proliferation was observed in front of M1-MoMφ treated with PS-liposomes. We believe that macrophage polarisation is a potential therapeutic strategy for T1D and other autoimmune diseases.

While our findings provide valuable insights to the field, we are aware of the limitations of the study. In the first place, the eight-day duration required for monocyte-to-macrophage differentiation severely augments the time required for obtaining a greater sample size. Another limitation is the heterogeneity of the subjects included in the study, inherent to the human condition [55]. Despite the narrow selection criteria established, variability coming from infections, genetics and other key environmental determinants could not be addressed. Finally, an important limitation is that macrophages derived *in vitro* do not fully capture the biological characteristics exhibited by Mφ undergoing natural differentiation *in vivo*. Besides, while our research utilised peripheral blood monocytes to generate various subclasses of Mφ, MoMφ represent only a fraction of the diverse population of tissue macrophages, which also includes foetal Mφ [56,57]. For that reason, we cannot extrapolate the results to *in vivo* conditions without further validation. However, and based on the knowledge generated through all preclinical studies with liposomes and biomimicry, it is reasonable to speculate that MoMφ will have a crucial role in self-tolerance recovery after PS-liposome administration. Recent studies provide mechanistic support for the role of liver macrophages in Ag-specific tolerance for a biomaterial platform that is currently being evaluated for clinical trials [58].

This work represents the initial effort to characterise how T1D MoMφ

respond to antigen-containing PS-liposomes, demonstrating that these cells emerge as primary targets for the induction of immune tolerance in ASITs. Furthermore, a subgroup within the T1D participants displayed abnormal characteristics upon response to LPS and IFN-γ what constitutes new evidence of the oddities attributed to myeloid cells in the context of this disease. Regardless of this fact, MoMφ from both groups engulfed PS-liposomes at similar rates and the nanoparticles were able to regulate the phenotype of non-inflammatory MoMφ, downmodulate the inflammatory state of M1 MoMφ and halt autologous T cell proliferation. Consequently, the use of multi-antigen-loaded PS-liposomes represents a tangible and innocuous strategy to restore tolerance in T1D. Moreover, PS-liposomes may benefit from the implementation of combined strategies with β cell regenerative drugs aiming at the cessation of the autoimmune attack and the reestablishment of a normal β cell mass [59]. Frequently, the development of ASITs and other immunoregulatory drugs blindly set the focus on DCs [60]. Collectively, our results put macrophages on the landscape of immune tolerance and encourage further investigation of their involvement in the restoration of immune homeostasis in T1D and beyond.

In summary, our results highlight the potential of PS-liposomes to target Mφ and make important contributions to understanding the mechanism of action of this preclinical immunotherapy in the arrest of antigen-specific autoimmunity.

5. Conclusions

This study investigates the role of Mφ in T1D and their response to antigen-containing PS-liposomes. The research identifies Mφ as key capturers of tolerogenic nanoparticles *in vivo* and defines the dynamics of capture of different human monocyte-derived macrophages, highlighting high rates of PS-liposome uptake particularly in the inflammatory subclass. Despite variations in MoMφ phenotype in T1D, PS-liposome uptake normalises marker expression and reduces pro-inflammatory cytokine secretion in M1-MoMφ. These findings support the benefit of PS-liposomes for inducing immune tolerance in T1D and helping advance the understanding of the mechanism of action of this preclinical immunotherapy.

Ethics statement

Humans. All the experiments were carried out in strict accordance with the principles outlined in the Declaration of Helsinki for human research and after the approval of the Committee on the Ethics of Research of the Germans Trias i Pujol Hospital. All participants gave their informed consent, and all procedures were carried out in accordance with approved guidelines.

Mice. All animal studies were approved by the institutional animal ethics committee.

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Contribution to the field statement

Our interest in understanding the role Mφ in the autoimmune destruction of insulin-producing β-cells in human T1D, as well as in antigen-specific immune tolerance, drives us to focus in studying this subset of APCs. Given the crucial role of Mφ in autoimmunity and the success of immunotherapies, we have investigated their specific particularities in T1D and their response to phosphatidylserine (PS)-liposomes, innovative nanoparticles designed to restore tolerance in experimental autoimmunity. This study reports, for the first-time, phenotypic alterations in Mφ from patients with T1D that do not affect their phagocytic activity nor their ability to induce tolerance in response to PS-liposomes. These results align with the tolerogenic potential of PS-liposomes, offering new insights into the not yet fully understood alterations of myeloid cells in the context of T1D and their role in the abrogation of autoimmunity following antigen-specific immunotherapy. The novel data reported herein contributes to a better understanding of the pathophysiology of the disease and enhances the potential application of immunotherapies to restore self-tolerance.

CRediT authorship contribution statement

Ivan Garcia-Loza: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **David Perna-Barrull:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **Eva Aguilera:** Methodology, Resources, Writing – review & editing, Conceptualization, Writing – original draft. **Lidia Almenara-Fuentes:** Investigation, Methodology, Validation, Writing – review & editing, Formal analysis. **Laia Gomez-Muñoz:** Resources, Writing – original draft, Writing – review & editing, Conceptualization, Methodology. **Daniela Greco:** Methodology, Writing – review & editing, Formal analysis. **Maria Vila:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Miriam Salvado:** Methodology, Resources, Writing – review & editing, Validation, Formal analysis, Writing – review & editing. **Montserrat Mancera-Arteu:** Methodology, Formal analysis. **Michael W. Olszowy:** Methodology, Resources, Writing – review & editing. **Jordi Petriz:** Formal analysis, Investigation, Methodology, Resources, Validation, Writing – review & editing. **Marti Dalmases:** Conceptualization, Writing – review & editing, Investigation, Supervision. **Silvia Rodriguez-Vidal:** Methodology, Resources, Writing – review & editing. **Bruna Barneda-Zahonero:** Conceptualization, Formal analysis, Investigation, Supervision, Writing – review & editing. **Marta Vives-Pi:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

MV-P and MD are co-founders of Ahead Therapeutics S.L., which aims at the clinical translation of PS-liposome immunotherapy for autoimmune diseases. LA-F, MV, DG, MS, MM-A, SR-V, and BB-Z are employees of this company. MWO works for Sartorius Stedim North America, Inc., which is in the business of selling live cell analysers and reagents. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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Abbreviations

Analysis of variance (ANOVA)
Antigen-presenting cells (APCs)
Antigen-specific immunotherapy (ASIT)
Area under the curve (AUC)
Body Mass Index (BMI)
C–C motif chemokine ligand 2 (CCL2)
Dendritic Cells (DCs)
Granulocyte-macrophage colony stimulating factor (GM-CSF)
Growth arrest-specific 6 (GAS6)
Human Leukocyte Antigen (HLA)
Indoleamine-2,3-oxygenase (IDO)
Interferon-γ (IFN-γ)
lipopolysaccharide (LPS)
Macrophage colony stimulating factor (M-CSF)
Macrophages (Mφ)
Median fluorescence intensity (MFI)
MER proto-oncogene, tyrosine kinase (MERTK)
Monocyte-derived dendritic cells (MoDCs)
Monocyte-derived macrophages (MoMφ)
3-hexanoyl-Nitrobenzoxadiazole Cholesterol (NBD)
Non-obese diabetic mice (NOD)
Peripheral blood mononuclear cells (PBMCs)
Propidium iodide (PI)
Programmed death-ligand (PDL)
Phosphatidylserine (PS)
Transforming growth factor beta (TGF-β)
Type 1 diabetes (T1D)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2024.103196.

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