Grant Agreement Number: 952183
Project Acronym: BOW
Project Full Title: Biogenic Organotropic Wetsuits
Start date of the project: November 1st, 2020
Duration: 48 months
Project Coordinator: Paolo Bergese (CSGI)
Project Officer: Ioannis Amarantos
Project Website: www.bowproject.eu
Project e-mail address: bow@csgi.unifi.it

D4.2. BOW products in vitro and ex vivo immunotoxicity/genotoxicity

DELIVERABLE INFORMATION	
Deliverable N°	4.2
Deliverable Title	BOW products in vitro and ex vivo immunotoxicity/genotoxicity
WP N°	4
Lead beneficiary	CNR
Contributing partners	CNR, CSGI, USC, UMORE
Type	Report
Lead authors	Paolo Colombo (CNR)
Due Submission Date	31/10/2023
Actual Submission Date	31/10/2023

TYPE		
R	Document, report	☒
DEM	Demonstrator, pilot, prototype	☐
DEC	Websites, patent filing, videos, etc.	☐
OTHER		☐

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

DISSEMINATION LEVEL		
PU	Public	☒
PP	Restricted to other programme participants (incl. Commission Services)	☐
RE	Restricted to a group specified by the consortium (incl. Commission Services)	☐
CO	Confidential, only for the members of the consortium (incl. Commission Services)	☐

STATUS	
Draft	☐
Final	☒

REVISION HISTORY		
Date	**Authors and Contributors**	**Comments**
13/07/2023	Noemi Aloi (CNR)	First Draft
05/10/2023	Paolo Colombo (CNR)	Review and editing of First Draft
05/10/2023	Noemi Aloi (CNR)	Second Draft
13/10/2023	Miriam Romano (CSGI), Andrea Zendrini (CSGI), Paolo Bergese (CSGI), Antonella Bongiovanni (CNR), José Rivas (USC), Massimo Dominici (UMORE)	Review and Editing of second draft
17/10/2023	Noemi Aloi (CNR)	Third Draft
20/10/2023	Paolo Colombo (CNR), Miriam Romano (CSGI), Andrea Zendrini (CSGI), Paolo Bergese (CSGI)	Review and editing of third draft
26/10/2023	Noemi Aloi, Paolo Colombo (IRIB CNR)	Fourth draft
31/10/2023	Miriam Romano (CSGI), Paolo Bergese (CSGI), Simonetta Tegliai (CSGI), Irene Trapani (CSGI)	Final Version

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

GLOSSARY OF TERMS	
Term	**Definition**
BOW	Biogenic Organotropic Wetsuits
WP	Work Package
NPs	Nanoparticles
EVs	Extracellular Vesicles
MBDs	Magnetic Bead Devices
MBDs-PDA	Magnetic Bead Devices functionalized with polydopamine
s.c. MBDs	Single core Magnetic Bead Devices
m.c. MBDs	Multi core Magnetic Bead Devices
MGAP-19	Selected prototype of s.c. MBDs
AP-20	Selected prototype of m.c. MBDs
EDT-MSCs	Human endometrial decidual tissue derived mesenchymal stromal/stem cells
MSC-EVs	EVs produced by EDT-MSCs
RBCs	Red Blood Cells
RBC-EVs	Red Blood Cells-derived EVs
lpMBDs	Liposome-membrane coated MBDs
PMA	Phorbol 12-myristate-13-acetate
LPS	Lipopolysaccharide
LAL	Limulus Amebocyte Lysate
qPCR	Quantitative polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
hIL-6	Human interleukin-6
hTNF-α	Human tumor necrosis factor-α
hACT	Human actin-β
PBMCs	Peripheral blood mononuclear cells

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

The statements made herein do not necessarily have the consent or agreement of the BOW consortium.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

INDEX

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

1. INTRODUCTION

1.1 OVERVIEW AND APPLICATION

Extracellular vesicles (EVs) are nanoparticles responsible for cell-cell communication and they are considered "nano-shuttles" as they transport lipids, proteins and nucleic acids, acting as intermediaries in both physiological and pathological processes. Their ability to act as vectors is due to their uniqueness in terms of membrane structure and composition.

The main objective of the BOW project is to explore and consolidate the possibility of creating a physical system (dressing cabin) to coat Magnetic Bead Devices (MBDs) with a lipid layer typical of extracellular vesicles derived from mesenchymal stem cells (MSC-EVs) and from vesicles from microalgae (*Tetraselmis chuii*) called nanoalgosomes.

The mechanism of action on the immune system differs depending on the origin and potential use of EVs. Therefore, it is necessary to clearly understand the EV effects on the immune system and perform toxicity tests (Holley & Dobrovolskaia, 2021).

The aim of T4.2 is to evaluate the immunological activity of the BOW products in specialized cell types of the immune system and their related soluble mediators. In particular, macrophages are believed to be among the first cell types that process foreign agents, mediating host inflammatory and immunological biological responses. These processes occur ubiquitously throughout tissues where nanomaterials are present. Thus, to understand exposure risks, it is critical to understand how cell-based reactions resulting from nanomaterial exposures can modulate inflammatory host responses. Monocytes are a type of white blood cells involved in immune system regulation. These immune cells circulate in the blood for several days before they enter the tissues, where they become specialized cells, called macrophages. They protect against viral, bacterial, and fungal infections, but in particular, they are involved in the strengthening of immune response and inflammation (Wang & Brenner, 2021). For this purpose, different *in vitro* and *ex vivo* experiments were carried out on human PMA differentiated THP-1 macrophage cell line and human whole blood, respectively.

As reported in D5.4, *in vitro* and *ex vivo* immunology has been completed on MBDs, nanoalgosomes, and MSC-EVs. The same assays were also performed on liposome-membrane coated MBDs and RBC-EVs. The methodologies and overall results of the products mentioned above are discussed in the present deliverable (D4.2).

We performed cytotoxicity and immune response studies in THP-1 cell line exposed to different concentrations of BOW products and time points.

Cytotoxicity linked to hemoglobin release and basophil activation assay for the potential anaphylactic activity were carried out on human blood.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

1.2 HEALTH & SAFETY WARNING

All the technical procedures described in this document are performed by trained personnel, in safety laboratory conditions. All workers have access to personal protective equipment. Regarding the experiments on human whole blood, the study was approved by the local Ethics Committee (Comitato Etico Palermo 1, 24 February 2021, resolution n. 02/2021.

2. MATERIAL AND METHODS

2.1 CELL LINE CULTURE MAINTENANCE

The immunosafety of BOW products were investigated using a human immortalized cell line, which reproduces the innate immune response in a simplified fashion.

The human monocytic leukemia THP-1 cell line was maintained in culture with complete medium (cRPMI) consisting of RPMI 1640 medium, supplemented with heat-inactivated 10% Fetal Bovine Serum (FBS) and 1% antibiotics. THP-1 monocytes were differentiated in THP-1 macrophages M(0) by treatment with 200 nM phorbol 12-myristate-13-acetate (PMA) for 72h at 37°C and 5% CO_2. Then, after 24h with cRPMI for the resting stage, THP-1 M(0) cells were treated with MBDs, EVs (nanoalgosomes, MSC-EVs or RBC-EVs), and liposome-membrane coated MBDs. .

2.2 *IN VITRO* EXPERIMENTS: METHODS OF TREATMENT

After differentiation in M(0) macrophages, the cells were seeded and treated with the BOW products at different concentrations and times. For cell viability assay (MTS assay), the THP-1 were seeded at 3.5 x 105 cells/ml in 96-well plate and treated for 24h, 48h and 72h with EVs or MBDs-PDA. For qPCR and ELISA, the cells were seeded at 5 x 105 cells/mL in 24-wells plate and treated for 24h (excluding MBDs-PDA treatments performed to feed D4.3, which included multiple time points (4h – 8h- 16h- 24h – 48h – 72h)).

2.3 ENDOTOXIN ASSESSMENT

The gel clot LAL assay provides an easy-to-read qualitative result indicating the presence or absence of endotoxin in the test sample. It is most often mentioned as the official referee test in pharmacopeial monographs.

Since endotoxin may interfere with the functional immunoassays, the potential presence of bacterial lipopolysaccharide (LPS) in the sample preparations was assessed using Limulus Amebocyte Lysate (LAL) test (Lonza, N594-03). So, different concentrations of MBDs-PDA were incubated with LAL reagent for 1h at 37°C for endotoxin detection following the manufacture's instructions; Internal standard positive and negative controls were used to compare the endotoxin levels in the MBDs-PDA samples.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

2.4 DETERMINATION OF CELL VIABILITY: MTS ASSAY

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium MTS assay, using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit, according to the manufacturer's protocol. The THP-1 M0 cells, seeded at a concentration of 3.5 x 10^5 cells/ml in 96-well tissue culture plates, were treated with different concentrations of BOW products at three different time points (24-48 and 72h). At the defined time point, the MTS reagent was added, and the cells were further incubated under the same conditions for 2 h. The absorbance of the dissolved formazan (Optical Density, OD) was measured with an automated microplate reader at 490 nm. Cell viability percentage was determined as follows:

$$\frac{O.D.treated\ cells}{O.D.control\ cells} \cdot 100 = viability$$

2.5 EVALUATION OF INFLAMMATORY IMMUNE RESPONSE

Transcriptional and translational analysis were performed to determine if BOW products could induce an inflammatory response in M(0) THP-1 differentiated macrophages. Quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) were applied to study the expression of the pro-inflammatory cytokines Interleukin-6 (IL-6) or Tumor Necrosis Factor α (TNF-α).

2.5.1 RNA ISOLATION, RETROTRANSCRIPTION AND qPCR

qPCR is a method that allows the amplification of nucleic acids in real time; it allows, in fact, the exponential synthesis of a DNA segment starting from a DNA template. It consists of several amplification cycles, each of which unfolds in three phases that take place at different temperatures. For qPCR analysis, THP-1 cells were seeded at concentration of 5 x 10^5 cells/mL in 24 wells-tissue culture plate and induced to differentiate into macrophages as previously described (Section 2.1). Then, cells were treated with BOW products for 24h. Supernatants were collected for the ELISA assays while total RNA was extracted using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Milan, Italy). The cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit with 2.5 µg of total RNA (Qiagen, Milan, Italy). The cDNA was diluted up to 100 µL, and Real-Time analyses were performed with an Applied Biosystems StepOnePlus™ Real-Time PCR System (ThermoFisher, Milan, Italy) and Sybr Green technology. Each sample contained 1-100 ng of cDNA in 2X Fast Sybr Green Master mix and 200 nM of specific Quantitect primer assay in a final volume of 20 µL (Qiagen, Milan, Italy). The expression of human interleukin-6 (hIL-6), human tumor necrosis factor α (hTNF-α) and of the housekeeping gene human actin-β (hACT) were analyzed, using the following PCR conditions: 95°C for 20 s, followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

2.5.2 ELISA

ELISA is an enzyme immunoassay that allows the detection and quantization of the molecule of our interest, exploiting the binding of this one, as an antigen, with the antibody, which is added during the procedure. IL-6 and TNF-α levels in conditioned media were determined using commercially available human IL-6 (Invitrogen, #BMS213INST) and TNF-α (Invitrogen #BMS223INST) ELISA kits according to the manufacturer's protocol (ThermoFisher, Italy). Medium supernatant and standards were added to the wells and incubated at room temperature for 1h on a horizontal shaker set at 700 rpm ± 100 rpm. Then, the plates were washed, and anti-IL-6 or anti-TNF-a conjugates were added into all the wells, and incubated for 1 h at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm. The plates were washed, and Chromogenic TMB was added to each well. The substrate solution begins to turn blue. After incubation for 15 minutes at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm in the dark, the stop solution was added, and the optical densities were read at 450 nm wavelength. The results of IL-6 or TNF-a were expressed as concentrations as pg/mL, using a standard calibration curve for each cytokine. The data were expressed as mean ± standard deviation (SD).

In addition, to study the potential M1/M2 polarization capability of the MBDs-PDA, Task 4.2 supported the activities of Task 4.3 of WP4. The aim was to validate a mathematical model for the toxicity risk of the BOW products, analyzing the effects of increasing MBDs-PDA concentrations (from 0.5 ug/ml to 10 ug/ml) at different time points (from 4h to 72h). Therefore, we evaluated the pro- and anti-inflammatory activity of MBDs-PDA after stimulation on THP-1 M(0) cells using the ELISA assay targeting TNF-α and IL-10 cytokines.

2.6 EX VIVO ASSAYS

In order to further analyze the immunological behavior of the BOW products, we studied the potential immunotoxicity of BOW products in two different *ex vivo* assays, the basophil activation assay and the hemolysis test using peripheral blood mononuclear cells (PBMCs) from healthy human blood. The study was approved by the local Ethics Committee (Comitato Etico Palermo 1, 24 February 2021, resolution n. 02/2021).

2.6.1 BASOPHIL ACTIVATION ASSAY

Basophils are a type of white blood cells whose function is linked to orchestrate the immune response to dampen the activation of allergen-mediated inflammation (Buhlmann basophil activation assay, Genoa, Italy).

The basophil activation assay was performed in heparinized peripheral blood from healthy subjects (n=3), using flow cytometry to detect the basophil markers CCR3 and CD63, to verify

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

the absence of potential immune reaction to the BOW products. A specific monoclonal antibody (anti-FcεRI), binding to the high-affinity IgE binding receptor, was used as positive control. Blood aliquots (100 µL) were incubated with MBDs-PDA, nanoalgosomes, and MSC-EVs dilutions in RPMI for 15 minutes at 37°C.

2.6.2 HEMOLYSIS TEST

Hemolysis is the destruction of red blood cells with hemoglobin release, which can occur in the presence of cell lysis.

The hemolysis test can provide valuable information about the hemocompatibility of NPs. Heparinized blood samples were obtained from three healthy human subjects. MBDs-PDAs were added to an 8% human erythrocytes solution and incubated at 37 °C for 30 min. The samples were centrifuged at 2000 x g for 5 min, and the supernatant absorbance was measured at 415 nm through a microplate reader to determine the percentage of hemolysis. Triton X-100 1% solution and 1X PBS were taken as 100% and 0% of hemoglobin release, respectively. Hemolysis percentage was determined as the ratio between the OD of treated cells and positive control cells.

3. RESULTS

3.1 IN VITRO AND EX VIVO IMMUNOTOXICITY OF MBDs-PDA

As already reported in D5.4, different experiments were carried out to assess the activation of the immune response of single core MBDs functionalized with PDA (MBDs-PDA) provided by USC and chosen due to their excellent biocompatibility in the presence of serum proteins (RP1). *In vitro* and *ex vivo* immunotoxicity of single core MBDs functionalized with polydopamine (MBDs-PDA) were published in Romano et al., ACS Biomater. Sci. Eng. 2023, 9, 303-317 (https://doi.org/10.1021/acsbiomaterials.2c00946).

3.1.1 ENDOTOXIN ASSESSMENT

Nanotechnology-based therapeutics are the new frontiers of medicine today. Moreover, they are characterized by a complex nature of intended components (therapeutic agents, targeting ligands, surface coatings) and undesirable contaminants such as microbes, endotoxins, and β-glucans. Contaminations are a key element in the interpretation of nanosafety studies because they can mask the true biological effects of NPs and immunological safety. All biological products are required to be tested for pyrogenicity. Indeed, in most cases, immunotoxicity and toxicity can be driven by impurities and pyrogenic contamination derived from the synthetic process. For these reasons, MBDs-PDA (hereafter referred to as SPIONs@SiO_2-PDA in the following figures) were analyzed for their endogenous content in LPS (**Table 1**). Two-fold serial

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

dilutions were tested for each sample until an endpoint was reached. The endotoxin concentration was expressed as EU/mL for a correlation between the concentration of endotoxins in our materials and the LPS concentration used in the internal standard, showing a small amount of endotoxin contamination in the range of concentrations used in the following biological assay.

Internal standard controls	NC (H_2O LAL)	0.0075 EU/mL	0.0125 EU/mL	0.03 EU/mL	0.06 EU/mL	PC 0.6 EU/mL
	-	-	-	+/-	+	+
MBDs-PDA (SPIONS@SiO2-PDA)	0.1 µg/mL	0.5 µg/mL	1 µg/mL	5 µg/mL	10 µg/mL	50 µg/mL
	-	-	-	+/-	+	+

Table 1: *Assessment of endotoxin contamination by LAL test..*

3.1.2 DETERMINATION OF CELL VIABILITY: MTS ASSAY

To evaluate the immunotoxic effects of MBDs-PDA on the innate immune response, M0 THP-1 macrophage-like cell line was incubated with increasing concentrations of MBDs-PDA (0.1 µg/mL, 1 µg/mL, 10 µg/mL, 50 µg/mL) for 24, 48 and 72 hours in complete RPMI-1640 medium. We analyzed the cellular metabolic activity as an indicator of cell viability using the MTS assay. Data showed that MBDs-PDA did not affect cell viability at 24 and 48 hours at all the tested concentrations, while we could observe a mild effect (<10%) at 72 hours when cells were treated with the highest concentrations (10 and 50 µg/mL).

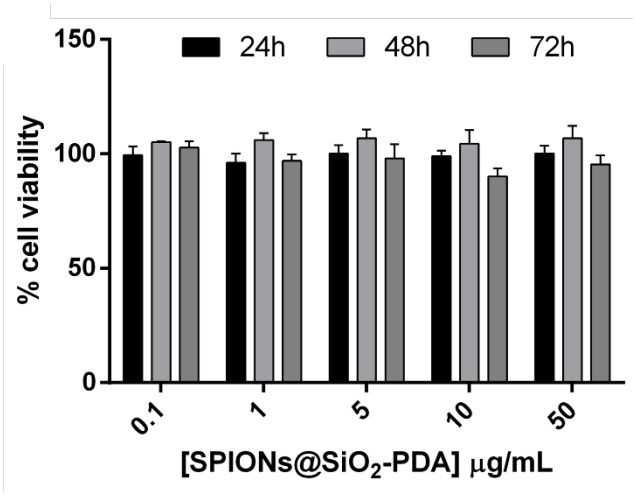

Figure 1: *Immunotoxicity measured by the MTS assay upon 24h, 48h and 72h. From Romano et al.,ACS Biomater. Sci. Eng. 2023*

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

3.1.3 ANALYSIS OF TNF-α PRODUCTION IN M(0) THP-1

Following the MTS data, where the THP-1 cell line did not show reduced cell viability at the highest concentration of MBDs-PDA at both 24 and 48 hours, we decided to study whether MBDs-PDA could induce a pro-inflammatory response on resting M(0) THP-1 macrophages-like cell line. TNF-α production was evaluated by ELISA assay after treatments with increasing concentrations of MBDs-PDA (0,1 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL) (Fig. 2). MBDs-PDA induced a detectable TNF-α production at the highest concentration (50 µg/mL) after 24 hours. Furthermore, at 48 hours, MBDs-PDA displayed a lower cytokine secretion and almost no cytokine production when used at a concentration below 10 µg/mL. Furthermore, the obtained results on TNF-α production were useful for the mathematical modelling as reported in D4.3 by T4.3.

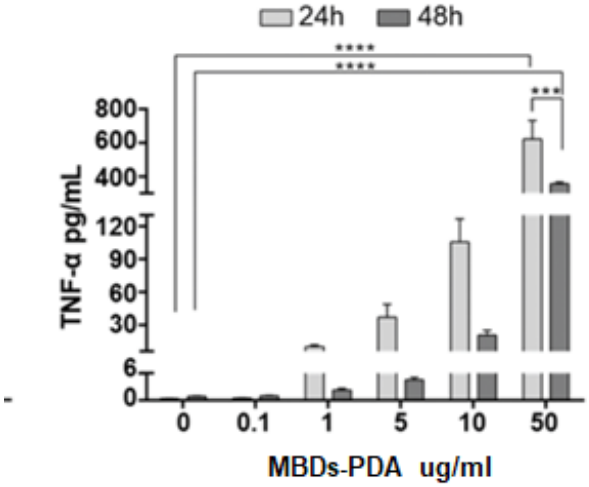

Figure 2 : TNF-α release measured by ELISA following 24 and 48 h exposure. Cytokine secretion in the culture medium is expressed in pg/mL. *From Romano et al.,ACS Biomater. Sci. Eng. 2023*

3.1.4 ANALYSIS OF THP-1 M1/M2 POLARIZATION AFTER MBDs-PDA TREATMENT

Developing a mathematical model for estimating the toxicity of the BOW products has been the objective of task T4.3 of WP4, and results are provided in deliverable D4.3. Here we report on the evaluation of M1/M2 polarization of THP-1 after treatment with increasing concentrations of MBDs-PDA (1,25 – 2,5 – 5 –10 µg/mL) at different time points (from 4h to 72h) that have been performed to feed T4.3 calculations and simulations. MBDs-PDA pro- and anti-inflammatory activity on THP-1 M(0) cells were assessed using the ELISA assay. The results showed a dose-response pick of the release of the pro-inflammatory cytokine TNF-α 8h after stimulation followed by a slow decrease (Fig. 3a); on the other hand, we observed an increase of the THP-1 anti-inflammatory cytokine IL-10 with a pick at 24 hours in a dose-dependent manner (Fig. 3b). These results demonstrated that macrophages' treatment with MBDs-PDA shifted the M(0) cells toward a pro-inflammatory status few hours after the treatment followed by an anti-inflammatory status in the latest ones.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

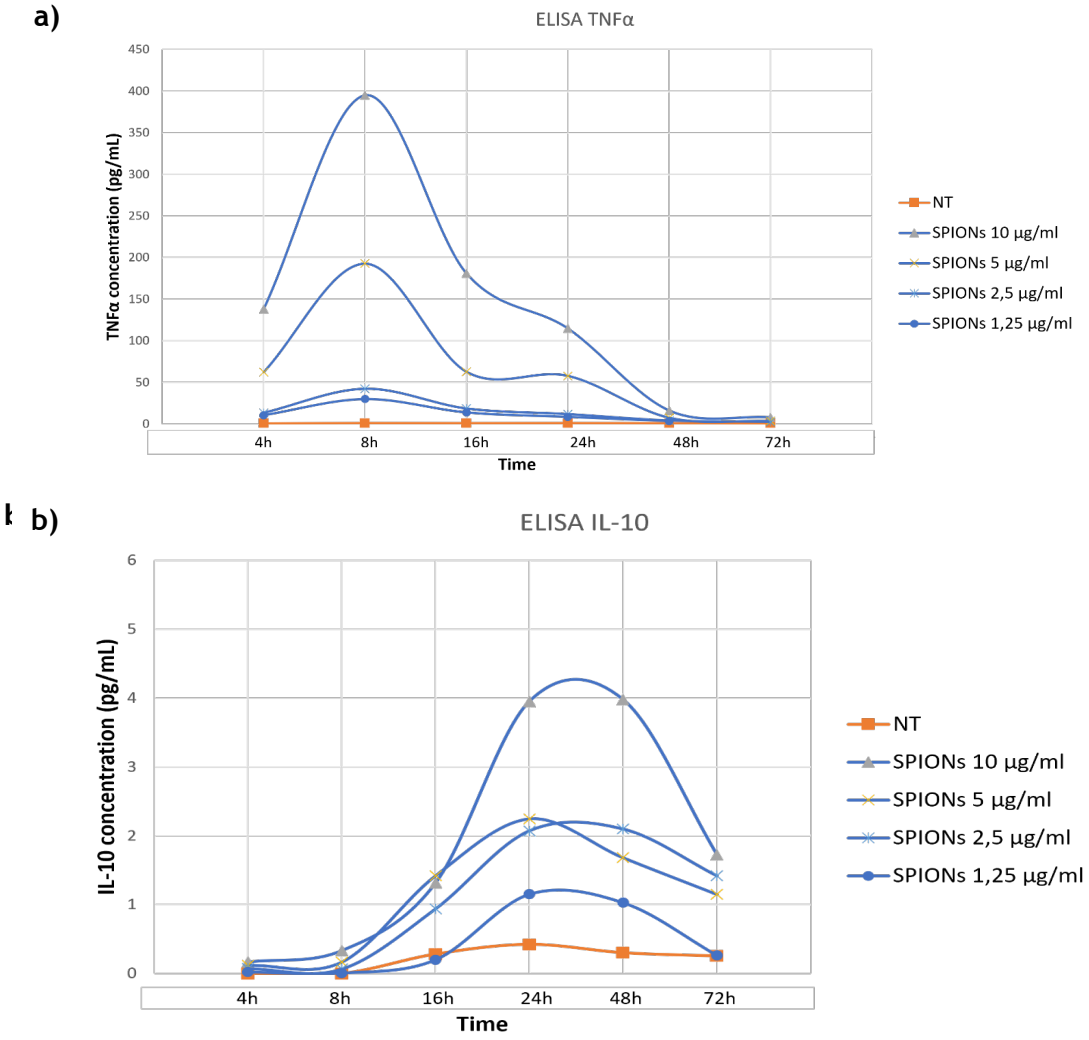

Figure 3: *Analysis of TNF-α and IL-10 release performed to feed T4.3 calculations and simulations.*

3.1.5 BASOPHIL ACTIVATION ASSAY

Blood from 4 volunteers were incubated with increasing concentration of MBDs-PDA, showing that the nanoparticles could not activate basophils *per se* from whole blood, with a percentage of activation similar to the negative control in all tested concentrations. These results demonstrated that MBDs-PDA cannot induce a hypersensitive reaction (Fig 4).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

			SPIONs@SiO_2-PDA			
Subject	**NC**	**PC**	**0.1 µg/mL**	**1 µg/mL**	**10 µg/mL**	**50 µg/mL**
#1	0.3%	85.9%	0.2%	1%	1.3%	0
#2	0.4%	89.9%	0.4%	0.9%	1.1%	1.6%
#3	0.9%	44.2%	0.9%	1.5%	2.3%	1.6%
#4	1.5%	93.7%	0.6%	1.6%	0.6%	0.8%

Figure 4: *Human basophil activation: NC negative control; PC positive control. From Romano et al.,ACS Biomater. Sci. Eng. 2023*

3.1.6 HEMOLYSIS TEST

To corroborate the low ex vivo immunotoxicity on PBMCs, we performed a red blood cell hemolysis test (*n* =3), showing that MBDs-PDA did not induce significant lysis of erythrocytes at the tested concentrations (Fig 5).

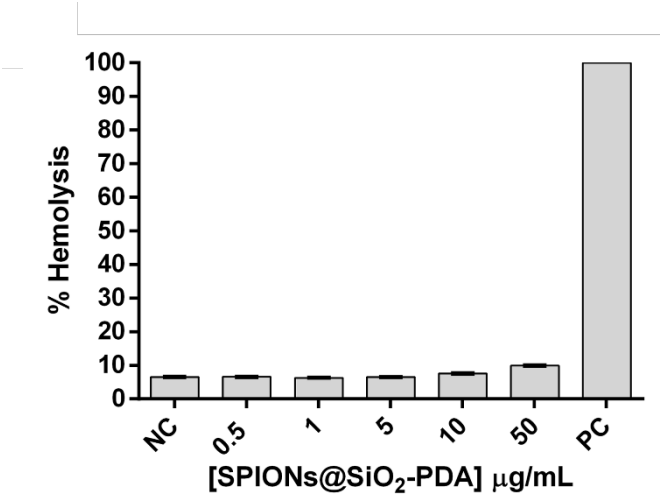

Figure 5: *Hemolytic potential of SPIONs in whole human blood sample. From Romano et al.,ACS Biomater. Sci. Eng. 2023. PC = positive control, NC = negative control.*

These data indicated that MBDs-PDA are biocompatible both *in vitro* and *ex vivo*, showing a mild cytotoxic action only at higher concentrations.

3.2 IN VITRO AND EX VIVO IMMUNOTOXICITY OF NANOALGOSOMES

Nanoalgosomes, produced and provided by IRIB-CNR, were isolated from marine microalgae *Tetraselmis chuii* as described within D1.2. Nanoalgosome biocompatibility was evaluated starting from *in vitro* experiments regarding immune response, followed by *ex vivo* assay. Four different batches (D13RI, D12R2, EA7R2, SG1R2) were analyzed using MTS assay, qPCR, and ELISA assay.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

3.2.1 DETERMINATION OF CELL VIABILITY: MTS ASSAY

After nanoalgosome treatment at different concentrations and time points, THP-1 M(0) did not show any significant cytotoxicity effect, showing a cell viability comparable to untreated cells (Fig 6).

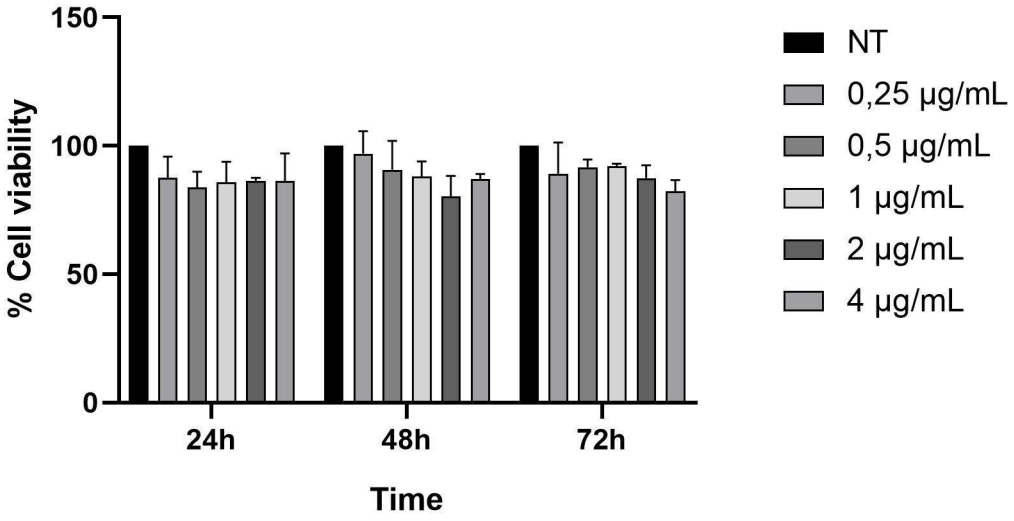

Figure 6: Immunotoxicity measured by the MTS assay upon *THP-1 treatments with nanoalgosomes after* 24h, 48h and 72h

3.2.2 ANTI-INFLAMMATORY ACTIVITY OF NANOALGOSOMES

In order to analyse the immunomodulatory activity of nanoalgosomes, THP-1 (M0) were pre-treated with 0,5 ng/mL of nanoalgosomes for 4 hours. Then, cells were stimulated with 10 ng/mL of LPS (Sigma Aldrich, Italy strain *E.coli* 0127:B8) to induce an inflammatory response for 20 hours. MTS cell viability assay was performed, to exclude any cell viability effects in our experimental conditions, showing that the LPS/Nanoalgomes treatment did not affect THP-1 cell viability (Fig. 7a). After that, gene and protein expression of interleukin-6 were monitored using qPCR and ELISA test, respectively (Fig. 7b-c). We demonstrated that nanoalgosomes promoted a reduction of IL-6 production in THP-1 M(0) treated with LPS, showing an anti-inflammatory activity.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

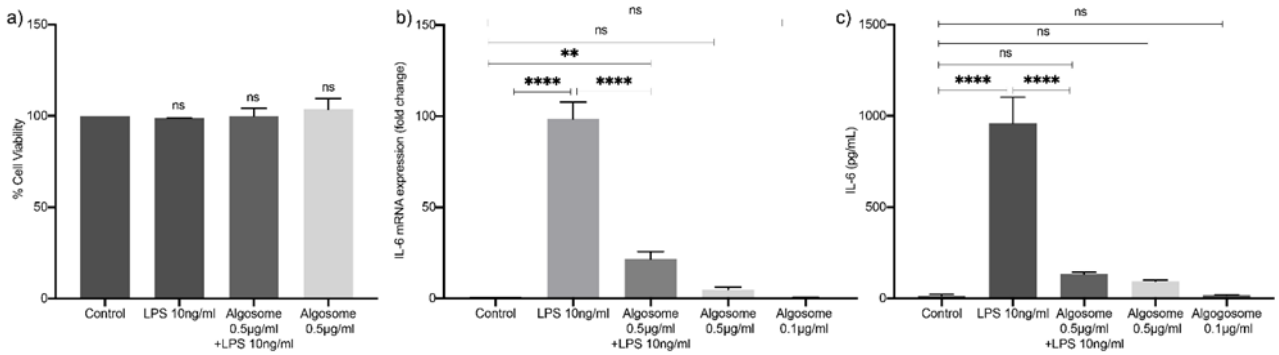

Figure 7: *Anti-inflammatory activity of nanoalgosomes.* a) MTS Cell viability assay; b) qPCR (IL.-6); c) ELISA (IL-6)**.**

3.2.3 BASOPHIL ACTIVATION ASSAY

To verify the absence of potential hypertensive immune reaction to nanoalgosomes, we performed an *ex vivo* basophil activation test using whole blood from three healthy donors. Flow cytometry characterization showed no basophil activation following nanoalgosome treatments at different doses, with a percentage of basophil activation similar to the negative control, demonstrating that nanoalgosomes did not display the ability to induce basophil activation (Fig 8).

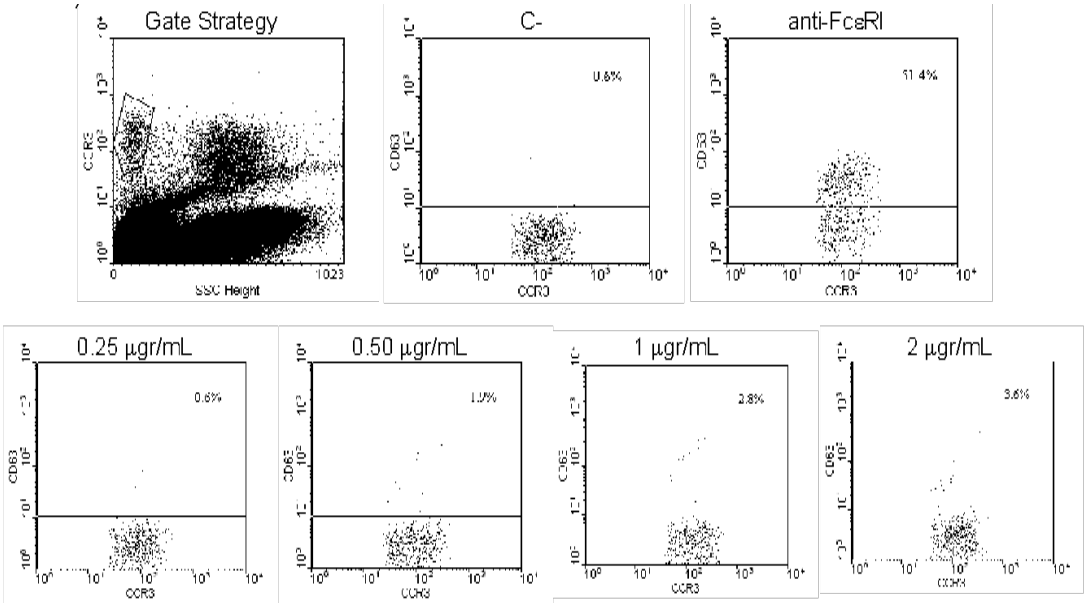

Figure 8: *Human basophil activation after incubation of* nanoalgosomes *with human peripheral blood MSC-EV. C- = negative control; anti-FceR = positive control.*

Our data show that nanoalgosomes do not have any cytotoxic effect on the PMA differentiated cell line THP-1, and they exert an anti-inflammatory effect in THP- 1 cell line in response to inflammatory insult (LPS) (Adamo et al 2023 doi.org/10.1101/2023.04.04.535547)**.**

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

3.3 IN VITRO AND EX VIVO IMMUNOTOXICITY OF MSC-EVs

MSC-EVs from Human endometrial decidual tissue-derived mesenchymal stromal/stem cells were produced as described in D1.1.

Their immunological response was evaluated starting from *in vitro* experiments in terms of immune response, followed by *ex vivo* assay.

3.3.1 DETERMINATION OF CELL VIABILITY: MTS ASSAY

Following the same strategies described for the other BOW products, THP-1 M(0) were treated with different concentrations of MSC-EVs (0,2 µg/mL – 2 µg/mL - 20 µg/mL) from 24h to 72h. As for the nanoalgosomes, MSC-EVs did not affect cell viability at each concentration and time points.

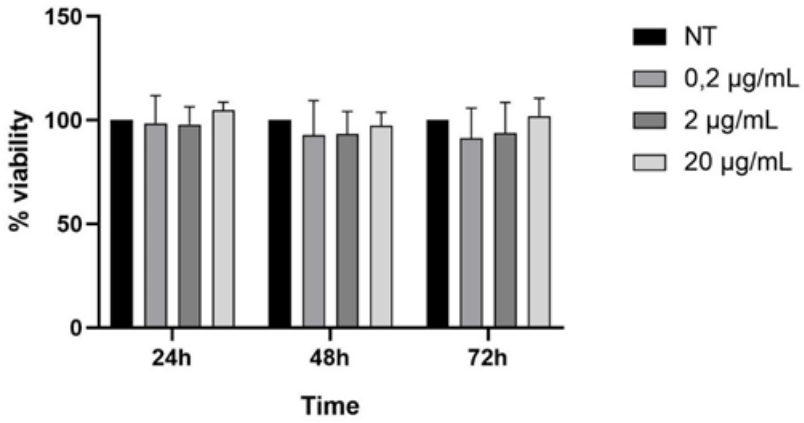

Figure 9: *Immunotoxicity measured by the MTS assay upon THP-1 treatments with MSC-EVs after 24h, 48h, and 72h.*

3.3.2 BASOPHIL ACTIVATION ASSAY

MSC-EVs were incubated with human peripheral blood from 3 volunteers, to assess the potential allergic activation. MSC-EVs did not activate basophils at all the tested concentrations, encouraging the absence of toxicity ex vivo (Fig. 10).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

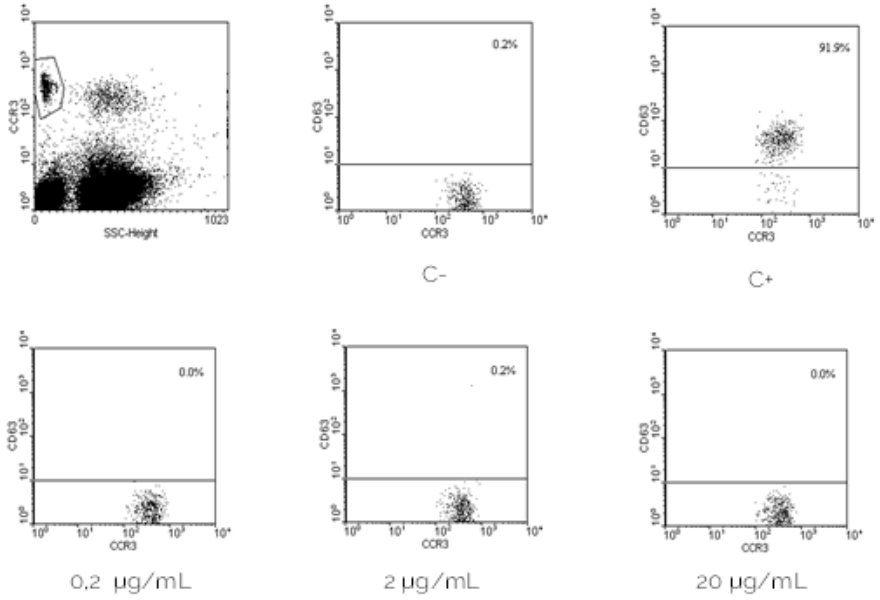

Figure 10: *Human basophil activation after incubation of* MSC-EVs *with human peripheral blood MSC-EV: C- = negative control; C+ = positive control*

However, experimental works on these samples has been limited due to the interruption of the production of MSC-EVs. Therefore, steps have already been taken to start testing RBC-EVs, provided by CSGI partner, as an alternative source of EVs with competitive characteristics and translational potential. Preliminary results on RBC-EVs are described in section 3.5 of this deliverable.

3.4 *IN VITRO* AND *EX VIVO* IMMUNOTOXICITY OF LIPOSOME-MEMBRANE COATED MBDs

As reported in D5.4, T3.e extra task was added to support T3.3 in producing the evMBDs and validate T3.1 and T3.2 approaches. T3.e provided an alternative bulk protocol to make liposome-membrane coated MBDs (lpMBDs) using osmotic shock. Specifically, MGAP19 MBDs were used as selected prototypes for single-core MBDs (s.c.MBDs), while AP-20 MBDs were used as selected prototypes for multi-core MBDs (m.c. MBDs) (D2.5, D2.6).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

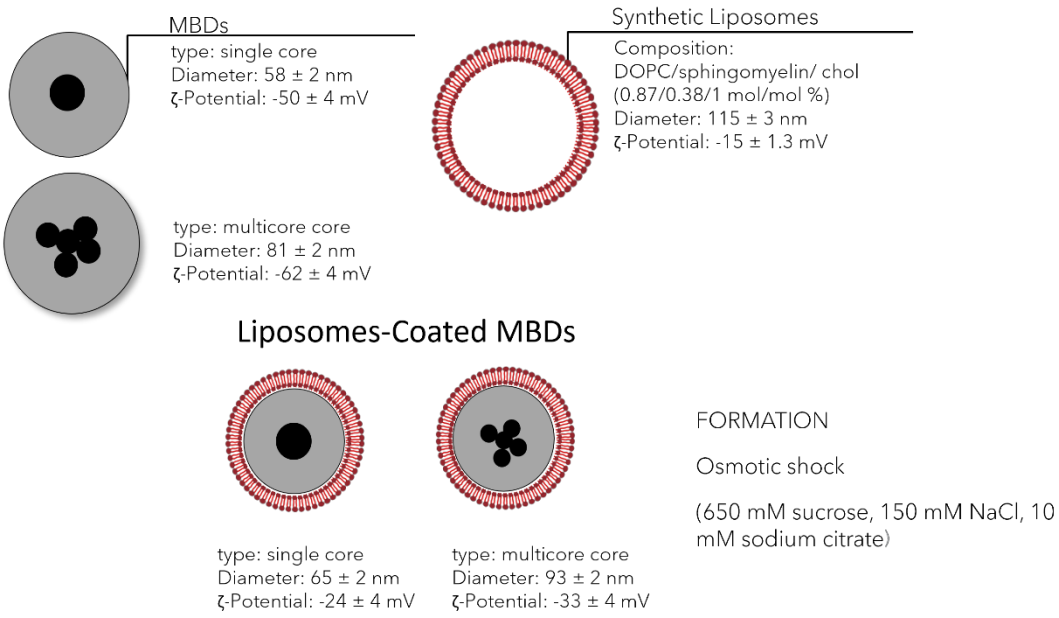

Figure 11: *Schematic representation of lpMBDs' production.*

The immunotoxic effects of lpMBDs were evaluated in THP-1 M(0) treated with increasing concentrations of lpMBDs or naked MBDs (0.1 µg/mL, 1 µg/mL, 5 µg/mL, 10 µg/mL, 50 µg/mL) for 24h in complete RPMI-1640 medium. After treatments with MGAP-19, we observed a mild protective effect when the MBDs were coated with the liposomal membrane compared to the naked ones; this effect was more marked and statistically significant in the cells stimulated with higher concentrations. These data demonstrated a potential reduction of immunotoxicity of lpMBDs.

For the analysis of the anti-inflammatory activity of lpMBDs, THP-1 (M0) cells were treated with different concentrations of AP-20 for 24 hours. Gene expression of pro-inflammatory cytokine TNF-α was monitored using qPCR. Preliminary data shows that lpMBDs did not promote inflammation in THP-1 M(0), showing an anti-inflammatory activity of these nanoparticles.

3.5 *IN VITRO* IMMUNOTOXICITY OF RBC-EVs

The use of Red Blood Cells-derived EVs (RBC-EVs) (provided by CSGI partner) was not scheduled in the work plan described in the GA. Still, it was introduced as a contingency plan to face the severe shortage (for about 5 months, from October 2022 to March 2023) of MSC-EVs due to the change of the partner in charge of their production. Because of the encouraging results, and of their translational potential – also recognized by the external scientific advisory board: "In the perspective of the member of the advisory board, the pursued "fall back strategies"

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

such as RBC-EVs could hold major potential for clinical application (see Annex 2)" – RBC-EVs have been thoroughly characterized (see Annex 1). They are now stably added in the pipeline of the project activities.

3.5.1 DETERMINATION OF CELL VIABILITY: MTS ASSAY

Red Blood Cells-derived Extracellular Vesicles (RBC-EVs) were provided from CSG partnersI. RBC-EVs immunotoxicity was evaluated in THP-1 M(0) incubating with increasing particle concentration of RBC-EVs (10^7 p/mL, 10^8 p/mL, 10^9 p/mL, 10^{10} p/mL) for 24h and 48h in complete RPMI-1640 medium. We did not observe any cytotoxicity effect at 24 and 48 hours), demonstrating their safety.

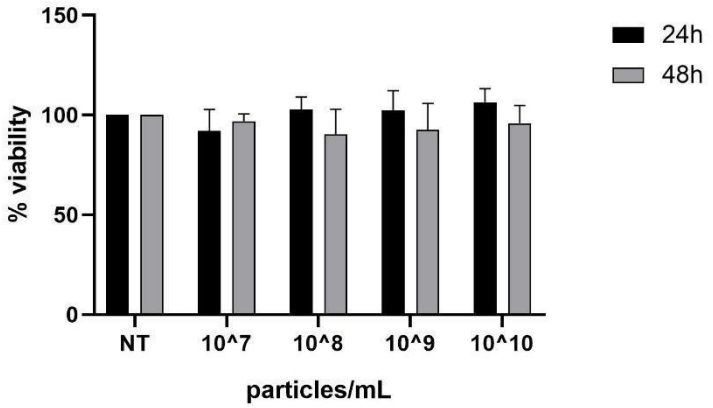

Figure 12: *Immunotoxicity measured by the MTS assay upon THP-1 treatments with RBC-EVs after 24h and 48h.*

4. CONCLUSIONS

The purpose of D4.2 is to study the activity of the BOW products in specialized immune system cell types. Data showed that nanoalgosomes, MBDs and MSC-EVs did not exert marked cytotoxicity both *in vitro* and *ex vivo*. In addition, RBC-EVs and lpMBDs have been also investigated, showing no direct cytotoxicity on a THP-1 PMA differentiated macrophage cell line. For lpMBDs, we observed that they display a better performance in *in vitro* MTS assays when compared to MBDs.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183

5. REFERENCES

- Holley, C. K., & Dobrovolskaia, M. A. (2021). Innate Immunity Modulating Impurities and the Immunotoxicity of Nanobiotechnology-Based Drug Products. *Molecules*, *26*(23), 7308. https://doi.org/10.3390/molecules26237308 ;
- Romano, M., González Gómez, M. A., Santonicola, P., Aloi, N., Offer, S., Pantzke, J., Raccosta, S., Longo, V., Surpi, A., Alacqua, S., Zampi, G., Dediu, V. A., Michalke, B., Zimmerman, R., Manno, M., Piñeiro, Y., Colombo, P., Di Schiavi, E., Rivas, J., … Di Bucchianico, S. (2023). Synthesis and Characterization of a Biocompatible Nanoplatform Based on Silica-Embedded SPIONs Functionalized with Polydopamine. *ACS Biomaterials Science & Engineering*, *9*(1), 303–317. https://doi.org/10.1021/acsbiomaterials.2c00946 ;
- Wang, Z., & Brenner, J. S. (2021). The Nano-War Against Complement Proteins. *The AAPS Journal*, *23*(5), 105. https://doi.org/10.1208/s12248-021-00630-9 ;
- Giorgia Adamo, Pamela Santonicola, Sabrina Picciotto, Paola Gargano, Aldo Nicosia, Valeria Longo, Noemi Aloi, Daniele, P. Romancino, Angela Paterna, Estella Rao, Samuele Raccosta, Rosina Noto, Monica Salamone, Irene Deidda, Salvatore Costa, Caterina DiSano, Giuseppina Zampi, Svenja Morsbach, Katharina Landfester, Paolo Colombo, Mingxing Wei, Paolo Bergese, Nicolas Touzet, Mauro Manno, Elia Di Schiavi, Antonella Bongiovanni (2023) Harnessing Nature's nanoSecrets: biocompatibility, biodistribution and bioactivity of extracellular vesicles derived from microalgae. bioRxiv 2023.04.04.535547; doi:https://doi.org/10.1101/2023.04.04.535547.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 952183