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D4.5: evBOW products in M. musculus

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GLOSSARY OF TERMS	
TERM	**DEFINITION**

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BOW	Biogenic Organotropic Wetsuits
EVs	Extracellular Vesicles
MBD	Magnetic Bead Device
NPs	Nanoparticles
evMBDs	EV membrane-coated MBDs
EDT-MSCs	Human endometrial decidual tissue derived-mesenchymal stromal/stem cells
dsRED- expressing EDT-MSC-EVs	Red fluorescent protein-expressing EDT-MSC-EVs
lpMBDs	Liposome-membrane coated MBDs
LPS	Lipopolysaccharide
L-IVM	Lung Intravital microscopy
BAL	Bronchoalveolar lavage

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INDEX

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1 Introduction

1.1 Executive summary

This deliverable describes the results obtained so far, within the activity of WP4, Task 4.5 (M7-M48), on the *in vivo* biocompatibility and pro- and anti-inflammatory effects in the lungs of mice of evBOW products upon instillation, towards application in pulmonary fibrosis. The BOW Consortium has identified the application of evMBDs for therapy and diagnosis in pulmonary inflammation and fibrosis as having significant potential. This experimental direction - not included in the Grant Agreement and detailed in the Periodic Technical Report for the second reporting period (RP2) - received positive feedback from reviewers, who supported it as a suitable necessary approach for the clinical application and implementation of BOW products. The evBOW products analyzed and described in this deliverable included EV-based raw materials, including human endometrial decidual tissue-derived mesenchymal stromal/stem cells (EDT-MSCs EVs), Tetraselmis chuii microalgae (nanoalgosomes), and Red Blood Cell-derived EVs (RBC-EVs), as well as MBDs and liposome-coated MBDs (lpMBDs). Experiments on evMBDs are ongoing and will be described in the project's final reports. lpMBDs have been used as a robust, repeatable, and largely available proxy of evMBDs, while RBC-EVs have been analyzed as a contingency plan to face the temporary shortage in MSC-EVs, as described in the Periodic Technical Report on the second reporting period (RP2). Testing of BOW products in *M. musculus* represents the final stage of an extensive *in vitro*, *ex vivo* and *in vivo* (in nematodes) characterization, described in deliverables D4.1, D4.2, and D4.4. Briefly, in D4.1, pre-screening and nanotoxicology studies were conducted using 2D and 3D lung tissue models at the Air-Liquid Interface (ALI), demonstrating minimal cytotoxicity and good biocompatibility. In D4.2, the immunotoxicity of BOW products was evaluated in a 2D *in vitro* cell model and *ex vivo*, which also showed low toxicity and favorable immune compatibility. Finally, in D4.4 highlighted that while most of the biological BOW products showed high biocompatibility, naked MBDs exhibited mild toxicity, which can be mitigated by liposome coatings, suggesting potential improvements for safe applications. Given this, the experiments were carried out in *M. Musculus*, as explained in the following sections.

1.2 Analysis of evBOW products lung biocompatibility in M. Musculus

At first, the evBOW products were investigated in mice for their cellular interactions and potential pro- or anti-inflammatory effects after instillation in the lungs of healthy mice. In the frame of the BOW project has been established the exogenous functionalization of the lpMBDs with folate to target the folate receptor β, which is overexpressed on activated lung profibrotic macrophages (D4.1 and D5.4).

To investigate the *in vivo* targeting of folate receptor β-expressing alveolar macrophages - which are key players in the initiation and resolution of inflammation and fibrosis - folate functionalized hybrids (folate-lpMBDs) have been examined in the newly established mouse model of Lipopolysaccharide (LPS) induced acute lung injury. This preclinical model was furthermore applied to elucidate the anti-inflammatory effects of nanoalgosomes *in vivo*. The acute inflammatory response in the lung is characterized by the influx of neutrophils from the blood circulation into the airspace across the endothelial-epithelial layer. Neutrophil recruitment in lungs after the application of evBOW products has been assessed and quantified by lung intravital microscopy (L-IVM), which was also used to detect

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evBOW product localization in the alveolar compartment of the lungs. Total and differential cell counts in bronchioalveolar lavage fluid (BALF) were analyzed to characterize the induced or lacking inflammatory response. Fluorescence microscopy of lung tissue sections was applied to localize evBOW products.

1.3 Health & safety warning

All the animal experiments shown have complied with the requirements reported in paragraph 5, "Ethics and security," section "5.1 ETHICS", subsection "5.1.3 ANIMALS" of the Grant Agreement.

2 Material and methods

2.1 evBOW products

The evBOW products used to assess their performance in *M. musculus* have been thoroughly characterized and pre-tested in the respective work packages. The related deliverables containing detailed information are referred to in the respective sections of this deliverable.

2.2 Lung application of evBOW products and endotoxin

Female C57BL/6N mice (8-12 weeks old, purchased from Charles River (Sulzfeld, Germany) were intratracheally instilled with 50 µl of evBOW product suspensions at concentrations and time points indicated in the respective result sections, according to established and published protocols (PMID: 28069010). The particle concentrations of evBOW products used in the *in vivo* experiments were aligned with those used for *in vitro* testing (D4.1 and D4.2). They matched the doses applied in EV biodistribution studies conducted in animals (PMID: 34194679). To induce endotoxin-mediated lung inflammation (acute lung injury, ALI), mice were intratracheally instilled with 0.1 µg of LPS (L2880-10MG, Sigma-Aldrich, Germany), as recently described (PMID: 28069010).

2.3 Lung intravital microscopy

Lung intravital microscopy (L-IVM) was performed, as recently reported by our group (PMID: 39364760). Briefly, deep anesthesia was induced using a triple compound of medetomidine (0.5 mg/kg body weight), midazolam (5 mg/kg body weight), and fentanyl (0.05 mg/kg body weight) (MMF) by intraperitoneal injection. Surgical sites (neck and left chest) were locally anesthetized with Bucain (50µg/site, Puren Pharma, Germany). Mice were positioned on a temperature-controlled heating pad to maintain the core body temperature at 37°C. anti-Ly6-G antibody for *in vivo* labeling of neutrophils was administered intravenously (i.v). The injection occurred 30 minutes before the L-IVM procedure. A tracheostomy was performed to insert a small catheter linked to a miniature rodent ventilator (MiniVent, Harvard Apparatus, Massachusetts, USA). The ventilator maintained 10 µl/g body weight for stroke volume and 150 breaths/min for breathing rate, coupled with a positive end-expiratory pressure of 2~3 cm H_2O, all administered with 100% oxygen throughout the experiments. The mice were then placed in the right lateral decubitus position, and a custom-made flanged thoracic suction window, furnished with an 8 mm glass coverslip (VWR, Radnor, USA), was inserted into a 5 mm intercostal incision through the

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parietal pleura between ribs 3 and 4 of the left chest. 20–25 mmHg of suction was used to immobilize the lung by a custom-made system consisting of a differential pressure gauge (Magnehelic, Dwyer Instruments, nc, USA) and a negative pressure pump (Nupro, St Willoughby, USA). Observations were facilitated using a VisiScope. A1 imaging system (Visitron Systems GmbH, Puchheim, Germany), equipped with a water dipping objective (20x, NA 1.0, Zeiss Micro Imaging GmbH, Jena, Germany), and a 16-color LED light source for fluorescence epi-illumination (pE-4000; CoolLED, UK). Throughout the experiment, a quadband filter set was used (F66-014, DAPI/FITC/Cy3/Cy5 Quad LED ET Set; AHF Analysentechnik AG, Tuebingen, Germany). The LED module 470 nm LED to excite Memglow green and Alexa488-labeled anti-Ly6G antibody, LED 550 nm for PKH-26 and DsRed, LED 635 nm for Cy5.5 and Memglow640, all at at 40% output power for 50 ms exposure time. Emissions were separated by a beam splitter (T 580 lpxxr, Chroma Technology Corp, Bellows Falls, USA), captured by two Rolera EM2 cameras, and processed using VisiView software (Visitron Systems GmbH, Puchheim, Germany). The images were calibrated to a scale of 2.5 pixels/µm using Fiji software (Rasband, W.S., U. S. National Institutes of Health). Neutrophil counts were assessed using the "Trackmate" plugin (119) in Fiji, with an estimated diameter of 10 µm.

2.4 Bronchoalveolar lavage (BAL) preparation and cell differentiation

Deeply anesthetized mice were humanely euthanized by abdominal aorta exsanguination. Dulbecco Phosphate Buffer Saline (D-PBS), instilled into the lungs through the tracheal cannula and aspirated by pulling on the syringe barrel, was used for bronchoalveolar lavage fluid collection. The cell pellet obtained from 10 ml of BAL was collected and counted with 0.2 % Trypan blue dye (Sigma Aldrich, Taufkirchen, Germany). For each cytospin, 3 x 104 BALF cells were utilized. Cytospin slides were stained with May-Grünwald-Giemsa staining (Sigma Aldrich, Taufkirchen, Germany) to identify macrophages, neutrophils, lymphocytes, and monocytes.

2.5 Immunofluorescence staining

Immunofluorescence of lung slices was performed as described in the M&M section of our recently published paper (PMID: 39364760).

3 Results

3.1 Nanoalgosomes

Based on literature (Adamo G. et al, 2021, PMID: 33936568, Adamo G. et al., 2024, PMID: 39097626) and information provided by BOW project partners producing and delivering nanoalgosomes (sEV, WP1, D1.2, and D1.4), and according to the *in vitro* biocompatibility testing performed at the Air-Liquid-Interface (D.4.1) and conventional *in vitro* and *ex vivo* immunotoxicity (D.4.2), 1 x 10^{10} (corresponding to 10µg total EV protein content) nanolgosomes have been applied into the lungs of mice *via* intratracheal instillation to monitor the pro-inflammatory potential of nanolgosomes by analysis of the bronchoalveolar lavage fluid (BALF). In accordance with the *in vitro* data (D.4.1, D.4.2), nanolgosomes did not induce an inflammatory response

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in the lung after 4h, 12h, or 3 days, as depicted in Fig. 1B, C and Fig. 1C. Total cell numbers recovered from BALF, as well as macrophage, neutrophil, lymphocyte, and monocyte numbers did not differ from those obtained in the vehicle control group (PBS 4h). The lung inflammatory response triggered by inflammatory stimuli (i.e., LPS) is characterized by the release of chemokines and upregulation of cell adhesion molecules resulting in a massive influx of neutrophils (peaking at 24h in case of LPS application) (Figure 1).

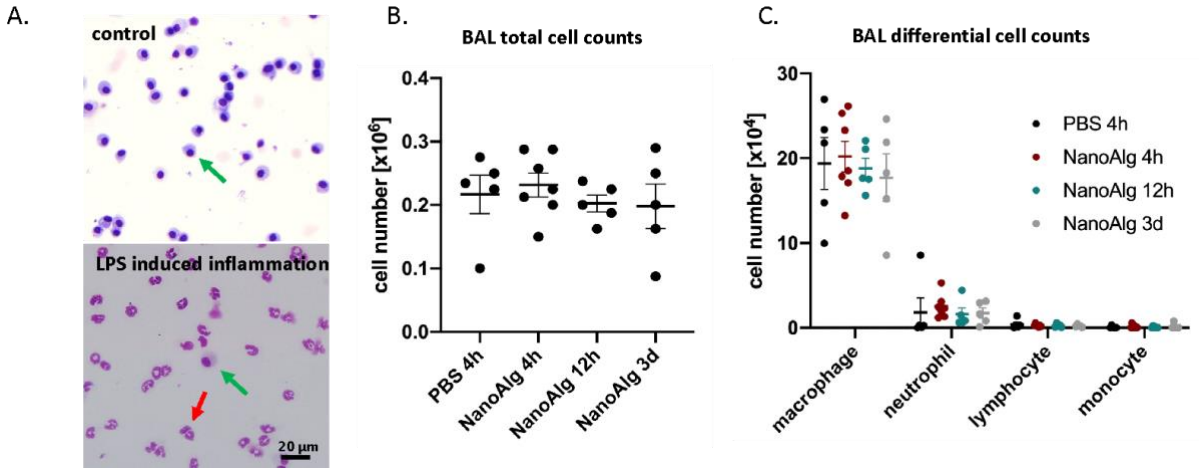

Figure 1 - Lung application of nanolgosomes does not cause inflammation. A. May Grünwald-Giemsa stained cytospin-samples of bronchoalveolar lavage (BAL) cells obtained from control mice and mice 24h after LPS-induced lung inflammation demonstrate hallmarks of lung inflammation. In healthy control lungs, only resident alveolar macrophages (green arrows) are flushed out, and infiltrated leukocytes (red arrows) are present in great numbers in BAL after LPS application. B. Quantification of total BAL cell- and C. differential cell counts (macrophage, neutrophil, lymphocyte, and monocyte) after instillation of 1 x 1010 nanolgosomes particles in 50µl PBS, or PBS (control) for 4h, 12h and 3d. May Grünwald-Giemsa-stained BAL cytospin samples were analyzed. (mean ± SEM, n = 5 mice/group).

3.2 MSC-EVs

Red fluorescent DsRed MSC-EVs from Human endometrial decidual tissue-derived mesenchymal stromal/stem cells (produced as described in D1.1) were tested due to their excellent biocompatibility determined in D.4.1 and D.4.2. Importantly, by application of fluorescent EVs (DsRed MSC-EVs) we could lay the ground for further detailed analysis of EV cellular interactions/uptake as well as distribution in the tissue, since DsRed MSC-EVs could be successfully detected using high-resolution lung-intravital microscopy as distinct fluorescent spots 4h upon application to the lung (Fig. 2 A, B), demonstrating the feasibility of the approach. Quantification of total BAL cell numbers confirmed the *in vitro* data since no increase in cell numbers could be detected compared to control (Fig. 2 C).

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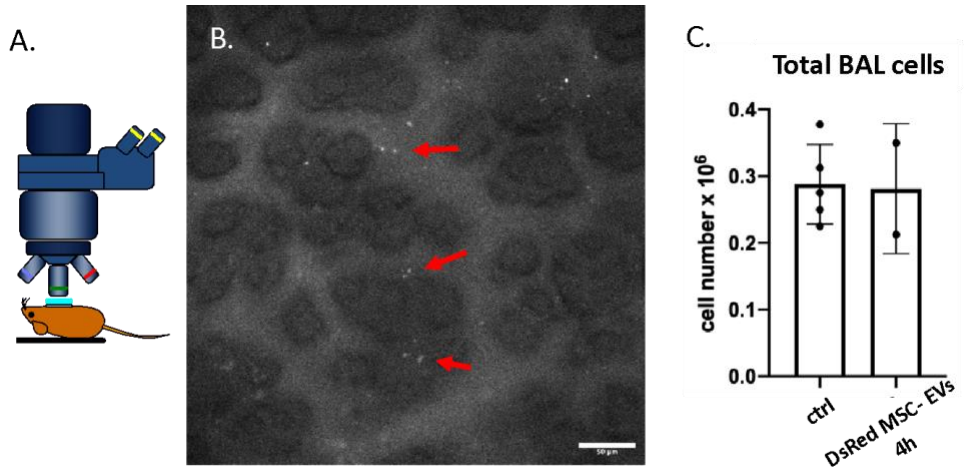

Figure 2 - **DsRed MSC-EVs can be detected by L-IVM upon instillation *in vivo* and do not cause inflammation A.** Schematic of Lung-intravital microscopy. **B.** Representative L-IVM image obtained 4h upon instillation of DsRed MSC-EVs (2x10^9 particles; 10µg). The fluorescent DsRed MSC-EVs are visible as distinct white spots (examples indicated by red arrows) in the alveolar region of the lung. Alveoli are discernible as black `holes´, in the surrounding interstitial tissue. **C.** Quantification of total BAL cell numbers after LIVM in May Grünwald-Giemsa-stained BAL cytospin samples. (mean ± SEM, n = 2-5 mice/group).

3.3 RBC-EVs

Red blood cell-derived EVs (RBC-EVs) have been included in the *in vivo* investigations since the Consortium had to face a severe shortage of MSC-EVs (for about 5 months, from October 2022 to March 2023). To cope with this, the Consortium decided to introduce RBC-EVs (provided by the CSGI partner) as an alternative to MSC-EVs with competitive characteristics and translational potential (D5.4). Four hours after instillation into the mouse lungs, fluorescent Memglow640 stained RBC-EVs were localized by L-IVM along alveoli walls, which indicates association with or uptake by alveolar epithelial cells (Fig. 3 A). Furthermore, the application of RBC-EVs induced neutrophil recruitment in pulmonary microvessels (Fig. 3 A, B) as assessed by L-IVM, as well as neutrophil influx into the airspace as evidenced by BAL analysis, thus indicating a pro-inflammatory potential of RBC-EVs in mouse lungs (Fig. 3 C, D, E).

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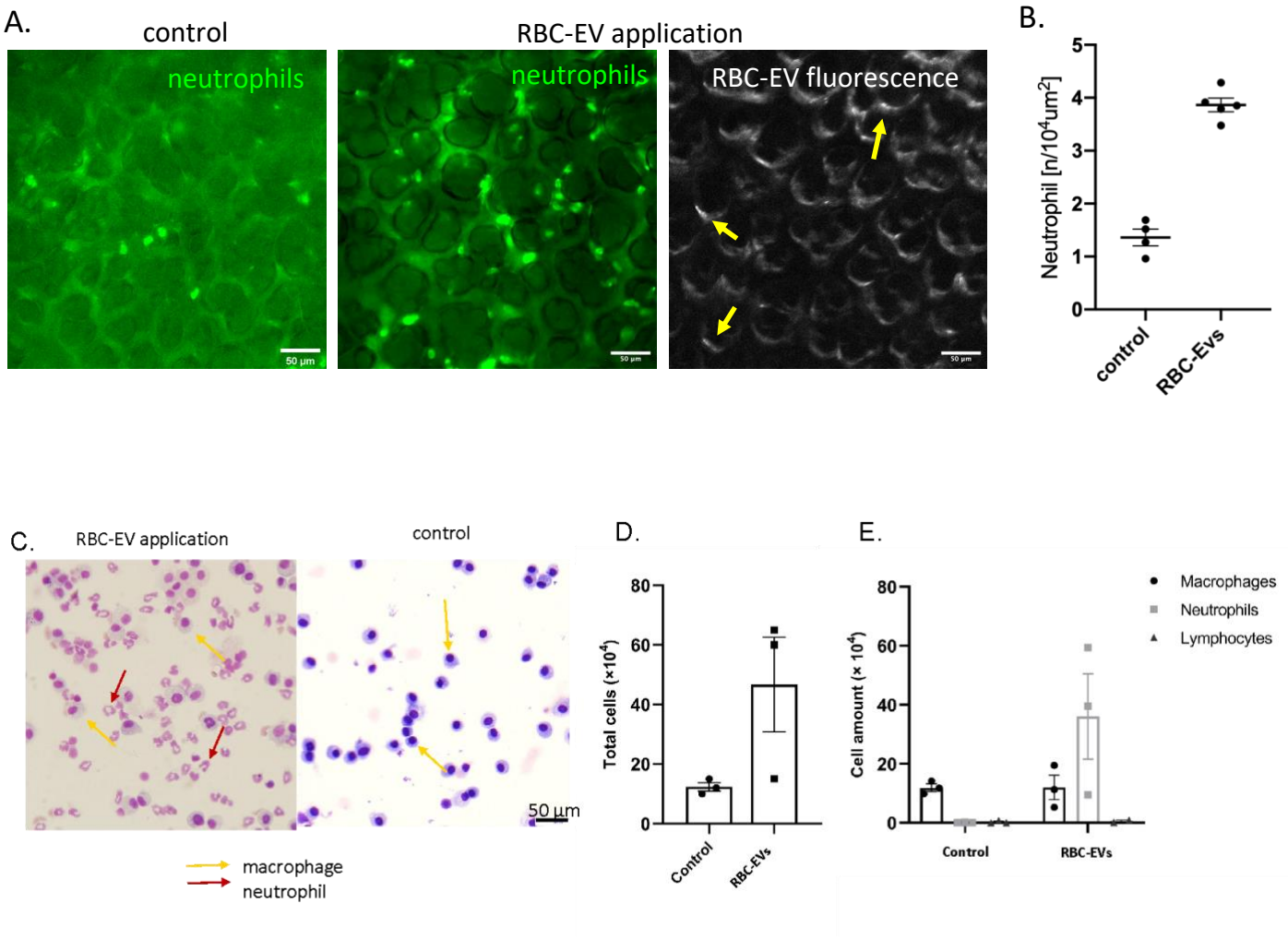

Figure 3 **Lung application of RBC-EVs results in neutrophil influx A.** Representative L-IVM image of neutrophil accumulations and RBC-EVs localization, obtained after 4h of 2.2 x 10^10 RBC-EVs/mouse *vs.* instillation of vehicle. Neutrophils are depicted in bright green, RBC-EVs in white. (examples highlighted by yellow arrows), RBC-EVs are Memglow640 stained. Neutrophils are mainly localized in the microvasculature surrounding the alveoli, which appear as `circles' with a diameter of ca. 30-40 µm. **B.** Quantification of RBC-EVs induced neutrophil recruitment detected by L-IVM to controls 4h after instillation. 4 mice/group, mean ± SEM. **C**. Representative May Grünwald-Giemsa stained cytospin samples of bronchoalveolar lavage (BAL) cells obtained 4h after 2.2 x 10^10 RBC-EVs in 50µl PBS or PBS (control) instillation. Meanwhile, in control lungs, only resident alveolar macrophages (yellow arrows) are flushed out, and infiltrated leukocytes (red arrows) are present in great numbers in BAL after RBC-EV application. **D.** Quantification of total BAL cell and **E.** differential cell counts (macrophage, neutrophil, lymphocyte, and monocyte) after instillation of RBC-EVs or PBS (control) for 4h. May Grünwald-Giemsa stained BAL cytospin samples were analyzed. (mean ± SEM, n = 5 mice/group). Scale bars: 50 µm.

3.4 MBDs AND lpMBDs

As outlined in D5.4, an additional task, T3.e, was introduced to assist T3.3 in generating the evMBDs and validate the approaches from T3.1 and T3.2. T3.e developed an alternative bulk protocol for creating liposome-membrane-coated MBDs (lpMBDs) using osmotic shock. In this process, MGAP19 MBDs (functionalized with rhodamine B isothiocyanate (RITC)), provided by the USC partner (D2.5, MGAP19; see also Acevedo et al, 2022, PMID: 35159800), were chosen as prototypes for single-core MBDs (s.c. MBDs). Here, we report the results

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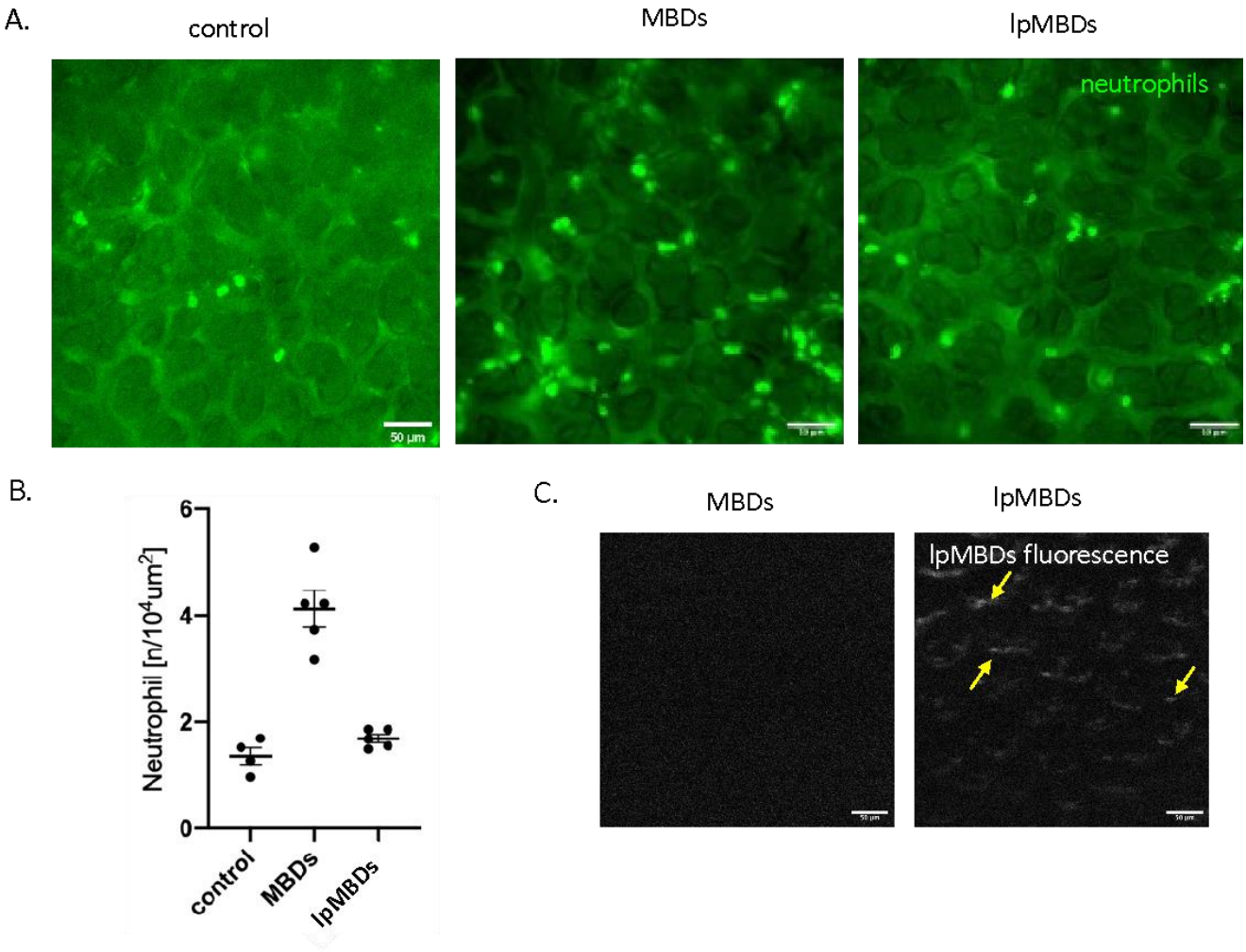
obtained with such a product. As reported in D.4.1, coating with liposome membranes reduced the cytotoxicity of MBDs (lpMBDs) at the Air-Liquid-Interface *in vitro*, compared to uncoated MBDs. In accordance with these results, lpMBDs application to the lungs resulted in comparable neutrophil levels to control conditions, whereas MBDs application induced neutrophil recruitment in pulmonary microvessels, as detected by L-IVM (Fig. 4A, B). RITC-fluorescence of MBDs could not be detected in these experiments, whereas deposited lpMBDs could be identified by their liposome-membrane Cy5.5 staining (made by CSGI partner), localizing at alveolar walls. On the level of total BAL cell counts, no differences were present in the treatment groups (Fig. 4D). However, as detected in the differential cell counts, the slightly increased numbers of airspace neutrophils obtained after the application of MBDs were reduced to control levels in the lpMBD group (Fig. 4E).



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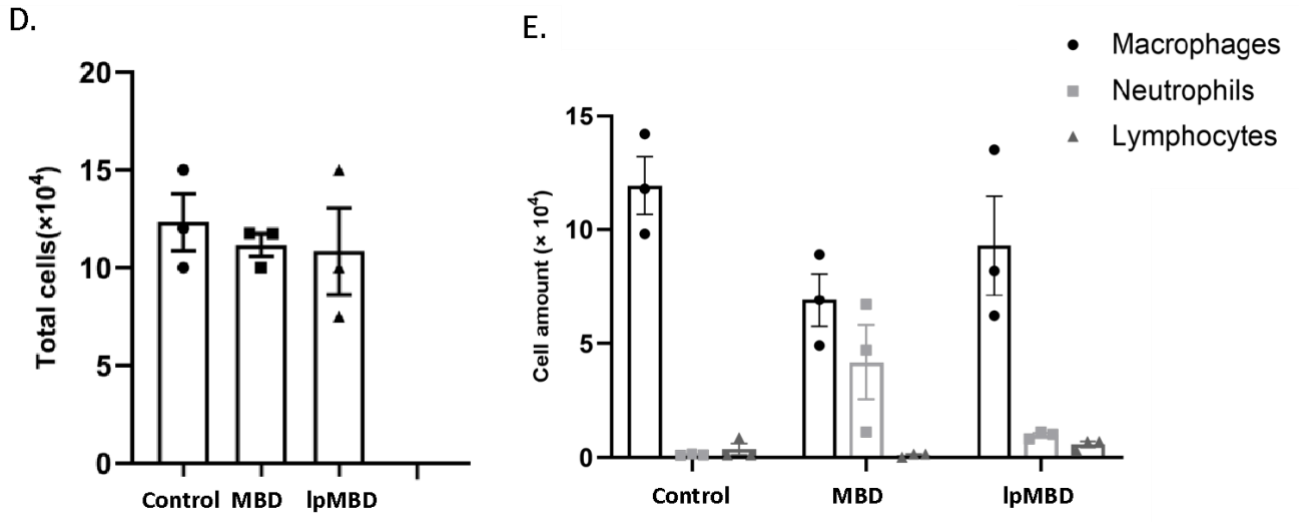

Figure 4 - **Lung application of magnetic bead devices (MBDs) versus liposome-coated MBDs (lpMBDs) A.** Representative L-IVM images of neutrophil accumulations obtained 4h after instillation of 2.5 x 10^10 MBDs (core contains rhodamine B isothiocyanate (RITC), or 2.5 x 10^10 lpMBDs (liposomes Cy5.5 stained) or vehicle. Neutrophils are depicted in bright green and are mainly localized in the microvasculature surrounding the alveoli, which appear as `circles´ with a diameter of ca. 30-40 µm. **B.** Quantification of neutrophil numbers detected by L-IVM after instillation of 2.5 x 10^10 lpMBDs, lpMBD or vehicle for 4h. 4-5 mice/group, mean ± SEM. **C**. L-IVM of MBDs and lpMBDs fluorescence in the lungs. MBD RITC fluorescence could not be detected, whereas deposited lpMBDs could be identified by their Cy5.5 staining (for better contrast depicted in white, examples highlighted by yellow arrows) in the alveoli. **D.** Quantification of total BAL cell numbers and **E.** differential cell counts (macrophage, neutrophil, lymphocyte, and monocyte) after instillation of MBDs, lpMBDs, or PBS (control) for 4h. May Grünwald-Giemsa stained BAL cytospin samples were analyzed. (mean ± SEM, n = 3 mice/group). Scale bars: 50 µm.

3.5 In vivo evaluation of anti-inflammatory effects of evBOW products

3.5.1 Establishment and validation of a lung disease mouse model of acute lung injury by lps application

To enable *in vivo* lung testing of anti-inflammatory effects of evBOW products during pulmonary inflammation and investigate macrophage targeting under physiologic and pathophysiologic conditions by functionalized evMBDs, an LPS mouse model for acute lung injury combined with L-IVM was established, and validated. Instillation of 0.1 µg induced within 1h the neutrophil recruitment in pulmonary microvessels (Fig. 5A, B), which led to severe pulmonary inflammation at 24h, characterized by the influx of neutrophils and monocytes, as assessed by BAL analysis (Fig 5 B).

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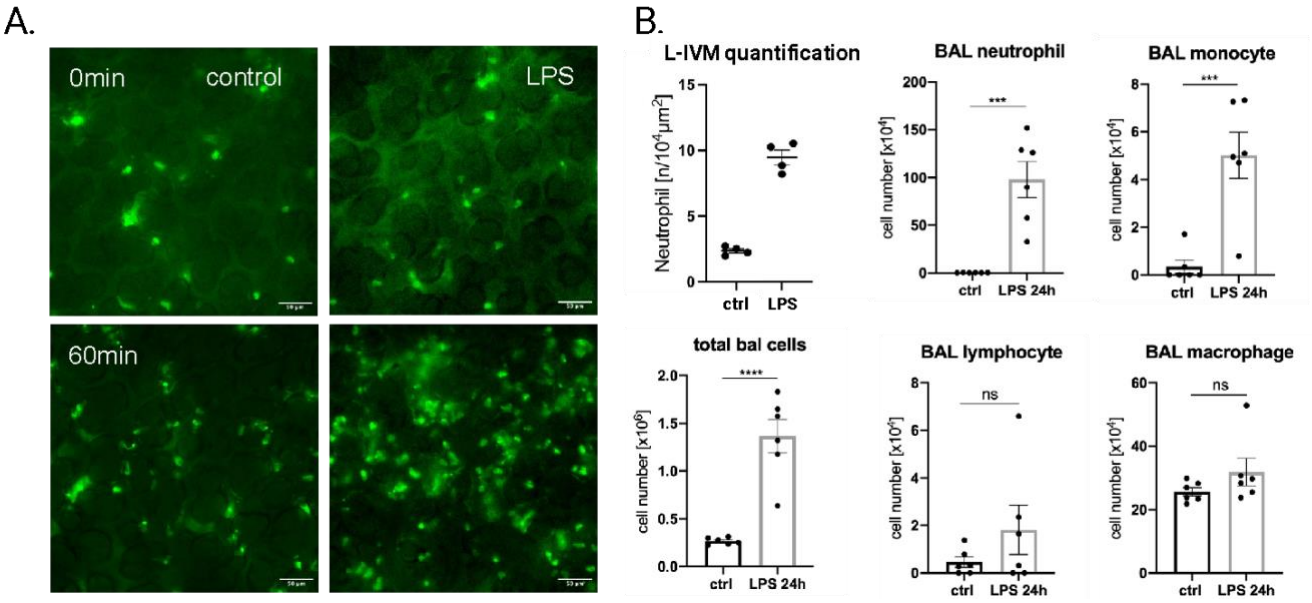

Figure 5 - **Establishment and characterization of the LPS animal model A.** Representative L-IVM images of neutrophils (green) in the alveolar region of the lung in control and LPS-treated (0.1 µg) mice at the start (0 min) and end of the recording (60 min) after instillation. **B**. Quantification of neutrophil numbers detected by L-IVM at 1h after LPS instillation. Total cell numbers and differential cell counts in BAL 24h after LPS instillation (0.1µg) show neutrophilic inflammation and influx of monocytes. May Grünwald-Giemsa-stained BAL cytospin samples were analyzed. (mean ± SEM, n = 6 mice/group, One-way ANOVA test, *** indicates P ≤ 0.001, and **** indicates P ≤ 0.0001). Scale Bars: 50 µm.

3.5.2 Anti-inflammatory effects of nanoalgosomes during lps-induced pulmonary inflammation

As published very recently by the consortium (Adamo G., Santonicola, P., Picciotto, S., PMID: 39097626) nanoalgosomes exhibit pronounced antioxidant and anti-inflammatory properties. Therefore, the anti-inflammatory effects of nanoalgosomes in the lungs of mice have been investigated by pretreating mice *via* instillation of 10 µg (total nanoalgosome protein content) of nanoalgosomes for 4h, followed by LPS treatment for 20h, along with the required control groups as depicted in Fig. 6 A.

Strong anti-inflammatory effects of nanoalgosomes in LPS-induced pulmonary inflammation could be detected. Nanoalgosome pretreatment reduced total BAL cell counts significantly (Fig. 6B), which was largely due to the reduction in neutrophil numbers in the airspace as deduced from differential cell counting (Fig. 6C). In histological stained lung slices derived from these mice, PKH-26 stained nanoalgosomes could be localized in CD68+ macrophages, in mice receiving nanoalgosome + LPS (lung inflammation) as well as in healthy mice, i.e. receiving nanoalgosome + PBS (healthy lungs) (Fig. 6D).

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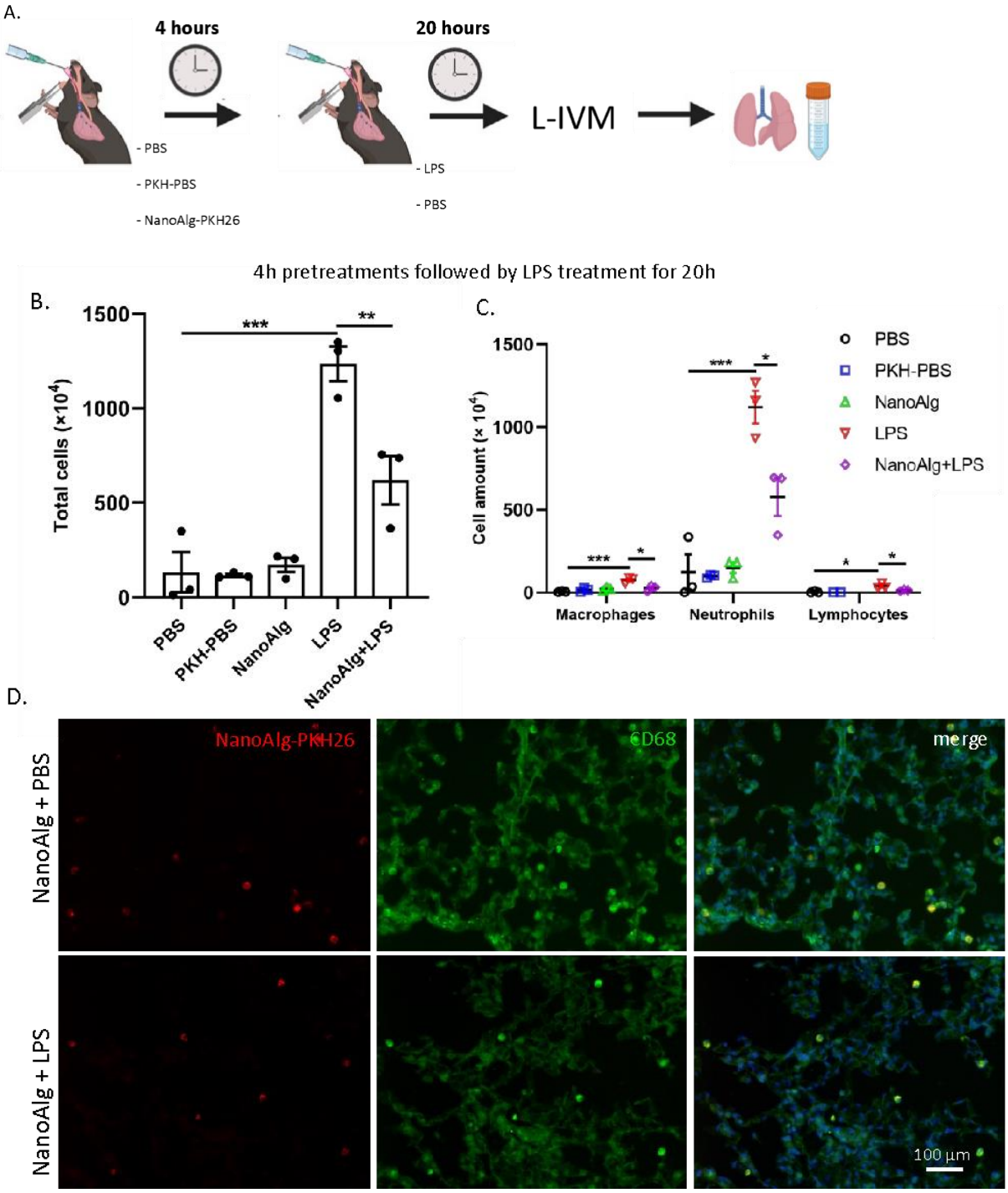

Figure 6 - **Strong anti-inflammatory effects of Nanoalgosomes in LPS-induced pulmonary inflammation A.** Experimental scheme**.** Mice were instilled for 4h with PBS (vehicle control), PKH26-PBS, (NanoAlg PKH labeling supernatant), or NanoAlg-PKH26 (10µg/mouse) for 4h, followed by instillation of LPS (0.1 µg) to induce pulmonary inflammation, or PBS, respectively, resulting in following experimental groups: G1 PBS 4h + PBS 20h; G2 PKH-PBS 4h + PBS 20h; G3 NanoAlg 4h + PBS 20h; G4 PKH-PBS 4h + LPS 20h; G5 NanoAlg 4h + LPS 20h. **B.** Quantification of total BAL cell numbers and **C**. differential cell counts (macrophage, neutrophil, lymphocyte, and monocyte) according to A. LPS-induced neutrophil recruitment to the lung is significantly reduced by pretreatment with nanoalgosomes. May Grünwald-Giemsa stained BAL cytospin samples were analyzed. (mean ± SEM, n = 3 mice/group, One-way ANOVA test, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$, and **** indicates $P \leq 0.0001$). **D.** NanoAlg-PKH are taken up by tissue-resident CD68+ macrophages in the lungs. D. Histology of CD68 expression (green) and NanoAlg-PKH62 localization in lung slices from mice instilled with NanoAlg-PKH (red), followed by instillation of PBS or LPS. In the merged image, DAPI is shown in blue (Scale bar: 100 µm).

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3.6 Cellular targeting by folate functionalization of lpMBDs

3.6.1 Analysis of folate-mediated lpMBDs targeting the lung

The exogenous functionalization of the evMBDs established in the frame of the BOW project (D5.4) includes the **development of folate**-functionalized evMBDs to target Folate receptor β, which is overexpressed on activated lung profibrotic macrophages. Therefore, a comparison of localization and cellular uptake of Memglow488 labeled, unmodified lpMBDs *vs.* folate-lpMBDs, obtained from partner MPG, was performed. Fluorescence microscopy on lung slices of mice receiving these evBOW products *via* instillation (4h) after pretreatment with LPS to enhance expression of FR β shows stronger MemglowGreen fluorescence, localized in macrophage-like cells, for the folate-lpMBDs.

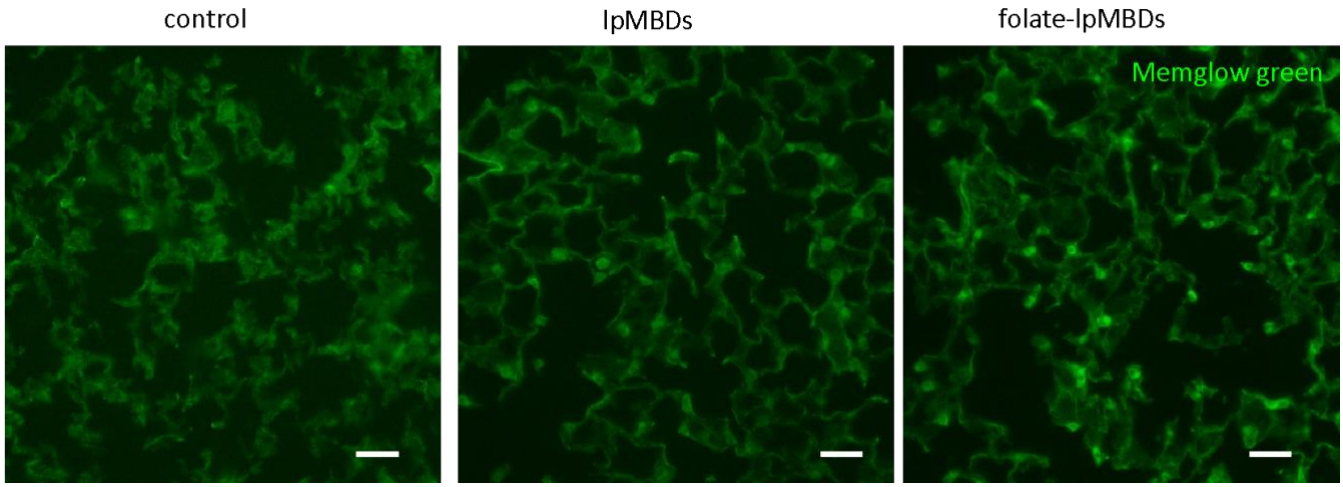

Figure 7 - **Folate-lpMBDs exhibit strong uptake in pulmonary macrophages.** Mice have been pretreated by instillation of 0.1 µg LPS (24h) to induce expression of folate receptor beta (FRβ) on macrophages in the lungs. After that, mice received via instillation 5 x 10^10 particles of Memglow488 stained lpMBDs or folate-lpMBDs, or vehicle (control). n=2 mice. Representative fluorescence microscopy images of lung slices of these mice show stronger Memglow green folate-lpMBD fluorescence in macrophage-like cells compared to Memglow green lpMBDs application. Note the strong tissue autofluorescence present under control conditions. n=2 mice; Scale bar: 50 µm

Due to strong tissue autofluorescence in the green channel, a new batch of orange-fluorescent folate-lpMBDs was synthesized and labeled using MemGlow590 (excitation: 590 nm, emission: 613 nm). Equal numbers of particles or vesicles—lpMBDs, folate-lpMBDs, liposomes, and folate-liposomes—were instilled into the lungs of LPS-pretreated mice. The use of MemGlow590 significantly enhanced the fluorescence signal of evBOW products compared to tissue autofluorescence, as shown in Figure 8A.

Interestingly, folate modification of the evBOW products decreased fluorescence intensity in the lungs. Since equal amounts of evBOW products were administered in all conditions, the observed reduction in fluorescence is likely attributable to the folate functionalization process. Although neither folate nor the reagents used for its conjugation are known to act as direct quenchers in the 600–620 nm spectral range, indirect quenching effects cannot be excluded.

Nonetheless, as illustrated in Figure 8B, only folate-lpMBDs exhibited clear cellular localization. These evBOW products accumulated in cells with the morphology and tissue distribution characteristic of macrophages, whereas unmodified lpMBDs appeared as isolated particles without distinct cellular association. This suggests that folate functionalization enhances macrophage targeting of lpMBDs within the lung tissue.

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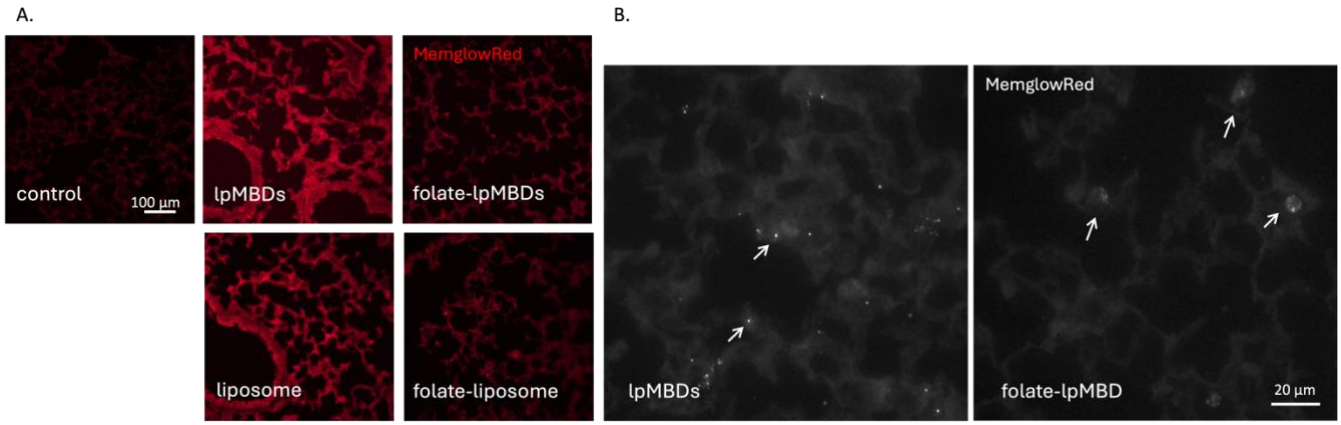

Figure 8 – **Memglow590** labeled **folate-lpMBDs confirm uptake in pulmonary macrophages.** Mice have been pretreated by instillation of 0.1 µg LPS (24h) to induce expression of folate receptor beta (FRβ) on macrophages in the lungs. After that, mice received via instillation 7.5×10^9 particles of Memglow590Red-stained lpMBDs, folate-lpMBDs, liposomes, folate-liposomes or vehicle (control). A. Representative fluorescence microscopy images of lung slices of these mice show strong MemglowRed fluorescence which is reduced in the folate-lmodified constructs. B. Confocal microscopy demonstrates cellular uptake and accumulation of folate-lpMBDs in macrophage-like cells, which is not observed for lpMBDs. Note the strong tissue autofluorescence present under control conditions. n=3 mice

4 Conclusions

This deliverable describes the results obtained to date on the biocompatibility and the **pro- or anti-inflammatory effects of the evBOW products in the lungs of mice** following instillation, toward potential application in **pulmonary fibrosis.** The pro- or anti-inflammatory effects have been evaluated in an LPS-induced acute lung injury model.

MSC-EVs, RBC-EVs, nanoalgosomes, MBDs, and lpMBDs have been tested and have shown to exhibit distinct interactions and effects on the mice lungs:

- pristine nanoalgosomes and MSC-EVs do not trigger significant inflammatory responses, underscoring their potential as safe therapeutic agents and as "wetsuit providers";

- in addition, pristine nanoalgosomes were found to reduce inflammation;

- to the contrary, pristine RBC-EVs were found to be pro-inflammatory;

- lpMBDs have reduced toxicity with respect to pristine MBDs. That is, **MBDs, when wrapped** (*viz.* cloaked, encapsulated) into a liposome membrane with a phospholipid composition that mimics the composition of the EV membrane, **are less toxic**. Remarkably, these results are i**n fair agreement with the other *in-vitro, ex-vitro, and in-vivo* (rapid invertebrate model) experiments in BOW,** (reported in D4.1, D4.2, D4.4);

- **lpMBDs functionalized with folate** to target profibrotic macrophages in the fibrotic lung have **shown higher targeting**. (Note: liposome membranes functionalized with folate are expected to be a closer mimic of the nanoalgosome membranes, testing of the hypothesis ongoing).

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