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Master's thesis

MacrophageDerived Extracellular Vesicles Impact OPC Differentiation in Multiple Sclerosis

Stéphanie Smets

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

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Macrophage-Derived Extracellular Vesicles Impact Oligodendrocyte Progenitor Cell Differentiation in Multiple Sclerosis

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Extracellular Vesicles Mediate Oligodendrocyte Progenitor Cell Differentiation in Multiple Sclerosis

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ABSTRACT

Progressive multiple sclerosis (MS) is associated with a failing maturation of oligodendrocyte precursor cells (OPC) into myelin-producing oligodendrocytes, an essential step towards remyelination. A potential therapeutic approach is to restore myelin by stimulating the OPC maturation. Myelin-phagocytosing macrophages play a contradicting role in the disease progression of MS by adopting an inflammatory or a wound-healing phenotype upon myelin internalisation, participating in both demyelination and remyelination respectively. However, the communication between wound-healing or inflammatory macrophages and OPCs regarding OPC differentiation remains elusive. Previous studies have defined the impact of the lipid cargo of extracellular vesicles (EV) on disease progression in several central nervous system disorders. EVs are mediators of intercellular communication by transporting biomolecules such as lipids. This project defines whether wound-healing macrophages stimulate OPC differentiation through the release of EVs, thereby promoting remyelination, and whether inflammatory macrophages inhibit OPC differentiation through the release of EVs. To prove this theory, we assessed the effect of EVs on OPC maturation *in vitro* by evaluating the differentiation status of the OPCs through immunostaining and qPCR. Secondly, the impact of EVs on lipid metabolism in OPCs is evaluated through qPCR. Moreover, nanoparticle tracking analysis, western blot, and transmission electron microscopy are applied for EV characterisation to ensure successful EV isolation. Our results revealed no significant impact of EVs on OPC differentiation and lipid metabolism. However, a trend is observed for the lipid metabolism between OPCs treated with wound-healing and pro-inflammatory EVs, possibly indicating an important interplay between macrophage-derived EVs, OPC differentiation, and lipid metabolism.

Multiple sclerosis (MS) is a chronic neurodegenerative disease of the central nervous system (CNS) which is the most common cause of non-traumatic neurological disability in young adults (1-3). Although MS has been investigated thoroughly, the fundamental cause remains elusive due to the heterogeneous and multifactorial nature of this disease (2, 4). The complexity of MS is supported by the importance of the interaction between environmental factors and genetic susceptibility which results in recurrent immune attacks in the CNS (2, 5, 6). Moreover, the heterogeneity in clinical course, therapeutic response, and pathological manifestation, further complicates the search for a single pathogenic mechanism (6). Ultimately, the immune system has been acknowledged as a major mediator to the pathology of MS by destroying neuronal myelin sheaths in the CNS (7).

The primary pathological hallmark of MS is the presence of neuroinflammation in which the chronic activation of the immune system and the subsequent infiltration of immune cells degrade

myelin sheaths leading to demyelinated lesions in the CNS (2, 3, 8, 9). These lesions may contain myelin debris or oligodendrocyte loss along with inflammatory cells, such as lymphocytes and macrophages (3, 5). The subsequent damage to axons and neurons, caused by demyelination and neuroinflammation, is followed by varying degrees of recovery in the majority of MS patients, which is classified as relapse-remitting MS (4, 5). Over time, the relapsing phase usually advances further towards irreversible neurodegeneration in which no periods of remission occur, termed as progressive MS (1, 7, 10). Rarely, this chronic progressive type is present from onset (2, 4, 5). Naturally, the varying anatomical lesions in MS lead to diverse clinical manifestations that typically range from spasticity and sensory loss to visual loss and bladder dysfunction (1, 5, 11). The substantial impact of these symptoms on the quality of life of MS patients highlights the need for curative therapies (2, 11).

Current treatments for MS rely on disease-modifying agents that reduce neuroinflammation in order to manage the course of MS (2). However, this treatment's effects are quite ineffective in the progressive type (1, 2, 12). A manner of acute management of MS relapses consists of administering high-dose corticosteroids which are associated with a fast functional recovery, but can only be given for a short period of time (2, 5, 10). Besides treatments to manage the disease course of MS, symptoms of MS can be targeted in order to improve the quality of life of MS patients (3, 11). While remarkable advancements have been made the past decade regarding disease management and increasing life expectancy, an effective therapy for progressive MS remains an unmet need (1, 7).

Throughout the years, remyelination has been the focus as a novel treatment strategy for future therapies to reverse neurodegeneration in progressive MS (2, 12). Remyelination is an endogenous repair mechanism that promotes the regeneration of myelin sheaths surrounding axons (7, 12, 13). As evidence has indicated that myelin does not only facilitate nerve conduction, but also protects against neurodegeneration by providing support and generating ATP, remyelination could restore functions and prevent further neurodegeneration (12). In the CNS, remyelination starts with the migration and recruitment of oligodendrocyte progenitor cells (OPC) followed by its differentiation into mature myelin-producing oligodendrocytes (7, 12, 13). Thus, stimulating remyelination through OPC differentiation could be an important therapeutic strategy for progressive MS in which the differentiation of OPCs is often impaired despite the presence of sufficient OPCs in MS lesions (2, 7). The differentiation of OPCs, along with OPC migration and proliferation, is fundamental for the remyelination process as priorly established in a study by Sim et al. (2002) and Arnett et al. (2004) in which repair and remyelination of demyelinated CNS lesions was impaired by a delayed OPC differentiation (14, 15). These results are confirmed by Mi et al. (2009) and Lombardi et al. (2019) in which myelin repair was enhanced and diminished by respectively stimulating and inhibiting the differentiation of OPCs into oligodendrocytes (16, 17). Hence, uncovering the reason of a failing OPC differentiation in progressive MS could pave the way towards a therapy that boosts the endogenous repair mechanism to relieve neurodegeneration, as a consequence of neuroinflammation, along with CNS function restoration. Considering the chronic immune activation and infiltration of immune cells in the CNS lesions as the primary pathological hallmarks of MS, the interplay between the immune system and OPCs may play a key role in remyelination failure (2, 3, 5, 16).

During neuroinflammation, macrophages infiltrate the CNS and accumulate in the inflammatory lesions, contributing to demyelination and tissue damage (18-21). Despite their detrimental contribution in disease progression in MS, macrophages have demonstrated an additional, yet contradicting, beneficial role in tissue repair, further complicating their role in MS pathology (18, 22). The heterogeneous properties of macrophages can be explained with the existence of a spectrum of phenotypes depending on the macrophages' activation status which is influenced by environmental signals. The most prominently polarised phenotypes are the classically activated M1 macrophages and the alternatively activated M2 macrophages (18, 19). M1 macrophages can be generated through stimulation with pro-inflammatory mediators such as lipopolysaccharide (LPS) and release a plethora of pro-inflammatory cytokines resulting in its

cytotoxic properties (19, 22). These pro-inflammatory mediators may include reactive oxygen species, nitric oxide, proteases, and glutamate, which are able to induce demyelination followed by neuronal death, and thus, are responsible for the destructive nature of M1 macrophages within demyelinating lesions (18, 19, 22, 23). This consequent neuroinflammatory incident creates a detrimental CNS environment which inhibits the maturation of OPCs into oligodendrocytes in progressive MS (24). Furthermore, treatment with anti-inflammatory mediators such as IL4 results in the polarisation to M2 macrophages characterised by the release of anti-inflammatory cytokines, such as IL10 and TGF-β (19, 22). Besides the production of anti-inflammatory cytokines, M2 macrophages also display regenerative properties that promote neuronal survival as well as OPC differentiation, which in turn enhances nerve regeneration, by secreting growth factors and trophic factors (18, 19, 22). Additionally, macrophages in MS lesions are in charge of myelin debris clearance through phagocytosis which is proven to be essential for CNS repair as myelin debris is shown to inhibit OPC differentiation (19, 20, 25). Hence, the infiltration and clearance by macrophages is crucial for generating myelin-producing oligodendrocytes and, thereby, for regenerating myelin sheaths (12, 18). The clearance of myelin debris is followed by the accumulation of myelin lipids upon myelin digestion. These so-called foamy macrophages display both M1 and M2 polarisations depending on myelin exposure (19, 21, 26). More specifically, *in vitro* experiments demonstrated that short term exposure to myelin converts myelin-phagocytosing macrophages towards a neuroprotective and regenerative phenotype, whereas long term accumulation of myelin skews myelin-phagocytosing macrophages towards a disease-promoting and inflammatory phenotype (20, 25). Ultimately, the exact role of macrophages in CNS repair in MS must be clearly understood in order to use their regenerative traits in a therapeutic approach. For this purpose, further clarification on the communication mechanisms with OPCs by which macrophages exert their inflammatory or reparative effects in MS is required.

Overall, macrophages are known to communicate with target cells either via direct cell-to-cell contact or via the secretion of cytokines and extracellular vesicles (EV) (27). EVs are nanosized particles surrounded by a lipid bilayer which are naturally secreted by nearly all types of cells to facilitate and mediate intercellular communication between local and distant cells (27-29). This form of communication relies on the ability of EVs to package bioactive cargo (including nucleic acids, lipids, and proteins), deliver it to a recipient cell, and alter the recipient's functions upon delivery through direct fusion between EVs and the plasma membrane, or EV internalization via endocytosis, macropinocytosis and phagocytosis (29-31). In turn, the altered function is comparable to the phenotype of the parental cell that had secreted the EVs. Consequently, EVs are thought to exhibit similar properties to their parental cell (27, 32, 33). For this reason, the participation of EVs in intercellular communication during disease pathology and tissue regeneration has been intensively explored in various diseases such as atherosclerosis, tumour growth in breast cancer, diabetes, and CNS disorders such as Alzheimer's disease, Parkinson's disease, and MS (27, 28). In general, EVs can be divided into 3 subtypes based on size, origin, and content: exosomes, microvesicles, and apoptotic bodies (28, 30). Exosomes are the smallest subtype, with a diameter of 30-150 nm, and are present in almost all biological fluids (30, 34). Exosomal vesicles are derived from the endolysosomal pathway, in which the inward budding of the cell membrane results in the formation of early endosomes which mature into multivesicular endosomes containing intraluminal vesicles that, upon fusion with the plasma membrane, are released as exosomes into the extracellular space (30, 34, 35). Microvesicles typically have a diameter ranging from 100 nm to 1 µm and are also present in almost all biological (30, 34). Microvesicles originate from the cell surface by direct outward budding of the cell's plasma membrane (30, 36). Lastly, apoptotic bodies are the largest subtype with a size ranging from 50 nm to 5 µm that are secreted by cells undergoing apoptosis (30). As of yet it remains difficult to accurately distinguish exosomes, microvesicles, and apoptotic bodies as the overlap in their size, as well as physiochemical and biochemical properties challenges the development of specific isolation techniques (28-30). Accordingly, EVs are practically classified into small EVs with a

diameter smaller than 100 nm or 200 nm, and large/medium EVs with a diameter larger than 200 nm (28, 37). As a consequence of the technical limitations to adequately isolate exosomes from microvesicles, the term 'extracellular vesicles' will be used throughout this article to refer to exosomes or microvesicles.

In MS, EVs have proven to be crucial participators in disease development through the propagation of pro-inflammatory signals, activation of monocytes, and enhancing neuroinflammation (38, 39). For instance, a study by Jy et al. (2004) showed that EVs derived from endothelial cells bind with high affinity to monocytes which activates and facilitates the transendothelial migration of monocytes across the protective blood-brain barrier (40). Another study by Verderio et al. (2012) demonstrated that EVs derived from reactive microglia propagate inflammation via pro-inflammatory signal delivery (41). On the contrary, EVs have also shown regenerative capabilities that enhance CNS remyelination (38, 42). The regenerative capacity of EVs was previously shown in a study by Kurachi et al. (2016) in which EVs derived from vascular endothelial cells promoted OPC survival and proliferation (43). The study from Willis et al. (2020) observed a great impact of astrocyte-derived EVs on OPC differentiation and in functional recovery from demyelinating lesions (44). In other words, EVs seem to exert a dual and non-negligible role in MS pathology, similar to the functions of macrophages. Hence, macrophage-derived EVs can serve as potential therapeutic targets or agents that stimulate remyelination by promoting OPC maturation (27, 36, 38-44). Therefore, macrophages may secrete EVs to mediate their immunomodulatory and regenerative properties, resulting in EVs with either disease-promoting or wound-healing effects depending on the phenotype of the parental macrophage (27, 32). In essence, it is anticipated that EVs, derived from myelin-phagocytosing macrophages exposed to myelin for 24 hours (mye^{24}-macrophages), are neuroprotective and induce OPC maturation followed by repair in CNS. On the contrary, it is expected that the exposure of myelin-phagocytosing macrophages to myelin for 72 hours (mye^{72}-macrophages) results in the release of cytotoxic EVs that counteract OPC maturation and contribute to disease progression.

Besides the phenotypes of the myelin-phagocytosing macrophages and the functions of their respective EVs, the effect of the lipid cargo of EVs should be of great value as well. The essential role of lipids in remyelination and demyelination is supported by the lipid-rich composition of myelin, consisting of approximately 70% lipids in which cholesterol is the major component (28, 45). Thus, when myelin debris is phagocytosed by myelin-phagocytosing macrophages, it is further processed into cholesterol metabolites and fatty acids (20). Considering myelin is heavily enriched in cholesterol, it is natural that the production of myelin sheaths requires a large amount of cholesterol and lipids for compact and correctly functioning myelin (46). As a result, the lipid metabolism and transport is increased during remyelination and clearance of myelin debris (45, 47, 48). To elaborate, OPCs undergo membrane expansion when differentiating into the extensively branched oligodendrocytes which requires an additional amount of cholesterol. Thus, cholesterol is needed for the maturation of oligodendrocytes and the myelination of axons (48). Furthermore, fatty acids as well as cholesterol are essential for an accurate myelination process, myelin growth, correct myelin composition, and stability of myelinated axons (45, 49). In addition, fatty acids are of high importance in oligodendrocytes to sustain remyelinating oligodendrocytes, to maintain survival of oligodendrocytes, and to increase the efficiency of remyelination as evidenced by Dimas et al. (2019). Here, the authors inhibited fatty acid synthesis which substantially reduced the amount of differentiated oligodendrocytes and axonal myelination (49). Altogether, EVs highly enriched in cholesterol metabolites and fatty acids potentially promote remyelination in MS through the stimulation of OPC differentiation.

This study aims to define the impact of EVs from myelin-phagocytosing macrophages on remyelination in MS (Fig. 1). We hypothesise that lipids within EVs released by macrophages exposed to myelin for short and long term will promote and suppress OPC differentiation, respectively. Furthermore, EVs derived from IL4- and LPS-stimulated macrophages are expected to have a similar impact on OPC differentiation. Through *in vitro* research, OPC differentiation is evaluated by

means of immunostaining and qPCR of mature versus immature oligodendrocyte markers and lipid metabolism markers. Although our results revealed no significant impact on OPC differentiation, a trend reveals higher levels of lipid metabolism markers in OPC cultures treated with wound-healing EVs. These findings indicate a potentially important interplay between macrophage-derived EVs, OPC differentiation, and lipid metabolism which provides more insights into remyelination in progressive MS.

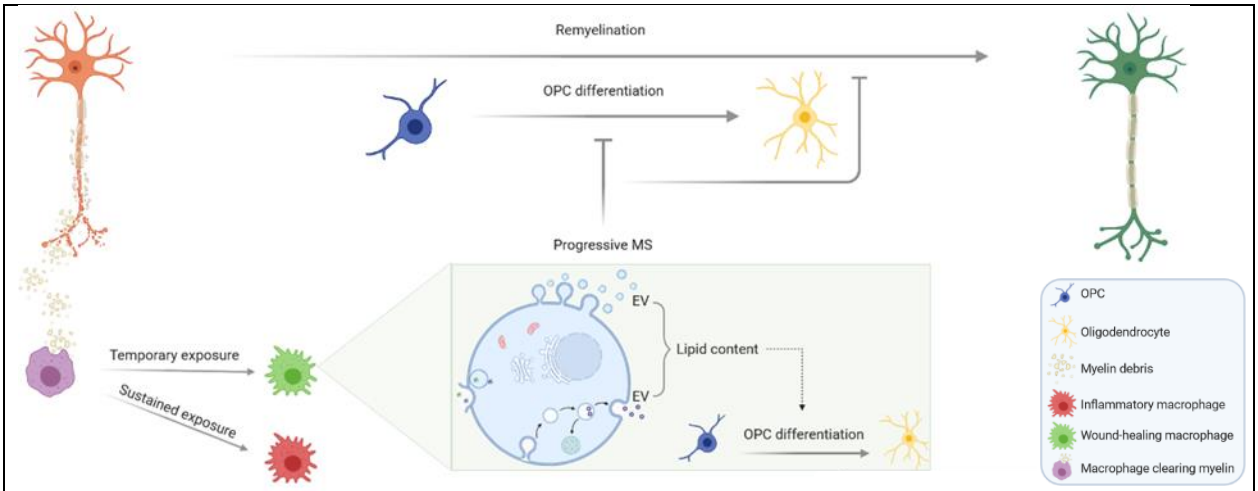


Fig. 1 - Lipids within extracellular vesicles (EV) released by myelin-phagocytosing macrophages stimulate oligodendrocyte progenitor cell (OPC) differentiation**.** In progressive multiple sclerosis (MS), endogenous repair mechanisms frequently fail. Macrophages exposed to high amounts of myelin are driven towards an inflammatory phenotype, whereas low amounts of myelin drive macrophages towards a wound-healing phenotype. Their exact role in remyelination in MS is unknown, but this study hypothesises that wound-healing macrophages stimulate OPC differentiation through the release of EVs, thereby improving remyelination. This research aims to define the impact of EVs released by inflammatory and wound-healing macrophages on OPC differentiation in MS.

EXPERIMENTAL PROCEDURES

BMDM Isolation – Murine bone marrow-derived macrophages (BMDM) are obtained by growing mouse femoral and tibial bone marrow cells, from 12 week-old C57Bl/6J female mice and sacrificing the mice by means of cervical dislocation (26). Once the femoral and tibial bones are collected, the bone marrow can be flushed out of the bones with the use of a syringe and a needle (> 25G) filled with 1xPBS. The cell suspension is centrifuged at 1600 rpm for 10 minutes. Lastly, the cells are plated by dissolving the pellets in FCS (Thermo Fisher Scientific) and adding it to BMDM medium (15% LCM, 10% FCS, and 0.5% P/S (Thermo Fisher Scientific) in RPMI1640 (Thermo Fisher Scientific)) which is transferred into a large petri dish. The BMDMs differentiate for 1 week at 37°C and 5% CO_2. After 1 week, the BMDMs are seeded at 500 000 cells/mL.

BMDM Treatment – T175 flasks containing 13.5 million cells are treated daily with 100 µg/mL myelin for either 24 hours or 72 hours, followed by an incubation period of 6 hours with MS-relevant pro-inflammatory (100 ng/mL LPS) and anti-inflammatory stimuli (20 ng/mL IL4) (Thermo Fisher Scientific).

Myelin Isolation – Mice are sacrificed by decapitation and myelin is isolated from brain tissue by means of density-gradient centrifugation. Then, the myelin protein concentration was determined by using the BCA protein assay kit (Thermo Fisher Scientific).

EV Production & Isolation – First, EV depleted medium is prepared after centrifugation for 16 hours at 100 000 rpm and at 4°C in order to start EV production, 5% LCM is added to the EV depleted medium which is added to the BMDM cell cultures. Then, medium with EVs is collected hourly throughout incubation at 37°C and 5% CO_2. Thereafter, EV isolation can commence by centrifuging the supernatant multiple times in the following order: 10 minutes at 300 rpm and 4°C, 10 minutes at 2000 rpm and 4°C, and 30 minutes at 10 000 rpm and 4°C. Finally, EVs are isolated as the supernatant is ultracentrifuged for 3 hours at 115 000 rpm and 4°C and filtered (10kDa). Then, size exclusion chromatography (Bio-Rad) is performed in which the fractions of small EVs (3.5mL to 5.5mL) are collected with the use of a column containing resin microscopic porous beads. Lastly, the EV sample is upconcentrated with the use of amicon filters of 10kDa (Merck Millipore) (50, 51).

Western Blot – SDS-PAGE gels are prepared with 12% resolving gel and 4% stacking gel. To start the separation of proteins, the samples are first lysed with RIPA Lysis and Extraction Buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Thermo Fisher Scientific). After adding sample buffer with beta-mercaptoethanol, the samples are incubated in a heat block for 5 minutes at 95°C. Next, the SDS-PAGE is run at 200V. Thereafter, the proteins are transferred onto a polyvinylidene difluoride transfer membrane (Immobilon) for 90 minutes at 350mA. In order to validate the purity of the EVs, the membrane is incubated overnight at 4°C with primary antibodies (1/1000) in 5% BSA in 1xTBS-T (1% Tween-20) (Sigma-Aldrich). The second day, the membrane is washed with 1xTBS-T (1% Tween-20) prior to the incubation of secondary antibodies (goat anti-rabbit HRP) (1/2000 in 1xTBS-T) for 1 hour at room temperature. Lastly, the membrane is washed with 1xTBS-T prior to visualisation with the use of the Sirius substrate kit (Advansta) and the ImageQuant (Amersham) device.

Nanoparticle Tracking Analysis – With the use of the NTA2.3 software (NanoSight), the diameter of EVs are measured according to their rate of Brownian motion. A camera detects the trajectory of each particle as it captures the light that is scattered when the particles are illuminated with a laser. Eventually, each particle's size is measured through the Stokes-Einstein equation resulting in EV size characterisation (52). The concentration of the samples is adjusted in order to detect 20-100 particles per frame.

Transmission Electron Microscopy – The spheric or cup-shaped morphology of EVs is evaluated with the use of transmission electron microscopy (TEM). To start, 20µL of EVs resuspended in PBS are placed on a clean Parafilm and formvar-nickel TEM slots are then placed on top of the droplets followed by an incubation period of 60 minutes to allow adsorption of the fluid onto the slots. Next, these slots are washed and fixed with 2% glutaraldehyde for 10 minutes. Thereafter, droplets of 2% uranyl acetate is added to the slots to incubate for 15 minutes. Finally, the slots are embedded in 0.13% methyl cellulose and 0.4%

uranyl acetate followed by examination with a transmission electron microscope at 120 kV (53).

OPC Isolation – OPCs are isolated by sacrificing postnatal day 0-2 C57BL/6 wild-type mice pups by means of decapitation. Then, meninges, blood vessels, and white matter are removed from the brain and the neonatal cortices are collected. Next, homogenised cortices are dissociated for 20 minutes with a papain-DNase solution (1/25, concentration papain = 20 Units/mL, concentration DNase = 50mg/mL). Centrifuge for 5 minutes at 300 rpm after inactivating digestion with cold DMEM (Gibco) and remove supernatant. Then, the mixed glial cells consisting of oligodendrocytes, microglia, and astrocytes are cultured in DMEM (Gibco) in flasks coated with 2mg/mL Poly-L-Lysine (PLL) for 14 days. Medium is replaced at day 3, 7, and 11, from day 7 onward 5µg/ml insulin is added. Thereafter, OPCs are isolated from the mixed glial culture by the shake-off method. Here, the flasks are agitated on a 37°C heated orbital shaker for 45 minutes at 75 rpm, followed by replacing of medium and overnight shaking of the flasks at 250 rpm for 16-18 hours. Then, the purified OPCs are plated in 24-well tissue culture plates, previously coated with 2mg/mL PLL. Next morning, remove medium and incubate on plastic petri dish for 25-30 minutes. Resuspend the cell pellet in differentiation, proliferation, or myelination medium (with 10ng/mL bFGF and 10ng/mL PDGF-AA) and culture the cells at a density of 150 000 cells/well for immunofluorescence staining and 250 000 cells/well for quantitative PCR. Lastly, EVs are added daily to the OPC cultures which are cultured at 37°C and 8.5% CO_2.

Immunofluorescence Staining – To start, the cells are fixated for 20 to 40 minutes with 4% paraformaldehyde (PFA). Next, the cells are blocked with 1% BSA in PBS-T (0.1% Tween-20) for 30 minutes. Primary antibodies (MBP: Millipore; O4: R&D Systems) are diluted in 1% BSA blocking buffer (MBP = 1/500; O4 = 1/1000) and are added to the wells followed by an incubation period of 4 hours at room temperature. Next, the wells are washed 1xPBS and the secondary antibodies, conjugated to Alexa Fluor 555 or Alexa Fluor 488 and diluted in 1xPBS (1/600), can be added to incubate for 1 hour at room temperature. Thereafter, the wells are washed with 1xPBS and DAPI is added. Next, the wells are washed 1xPBS and the small staining glasses can be mounted onto microscope glasses with the use of fluorescent mounting medium. The OPCs are then visualised with the Leica fluorescence microscope and quantified with ImageJ (Fiji).

RNA Isolation & Quantitative PCR – OPC samples are first lysed with QIAzol Lysis Reagent containing $1/5^{th}$ chloroform which is shaken vigorously for 15 seconds prior to centrifugation at 12 000 rpm for 15 minutes at 4°C. Then, the upper aqueous phase representing the RNA is harvested and total RNA is prepared using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. After RNA isolation, the RNA quality is determined using a NanoDrop spectrophotometer followed by cDNA synthesis with the use of qScript cDNA SuperMix (Bio-Rad) to reverse transcribe RNA. Lastly, 0.3µL of reverse and forward primers with SYBR Green are used per 5ng/µL cDNA sample to perform qPCR.

Statistical Analysis – Statistical analyses is performed using non-parametric Kruskal–Wallis test by ranks. Data were analysed using the GraphPad Prism software and presented as the mean ± SEM. Values of $P < 0.05$ are considered statistically significant.

RESULTS

Considerable evidence suggests that EVs play a dual and non-negligible role in MS pathology, similar to the functions of macrophages (38-44). As a result, it is anticipated that macrophage-derived EVs can serve as potential therapeutic targets that stimulate tissue regeneration by promoting OPC maturation (27, 32). This study aims to define the impact of macrophage-derived EVs on OPC differentiation, hypothesising that mye^{24}-EVs and IL4-EVs will have a stimulating impact on OPC differentiation whereas mye^{72}-EVs and LPS-EVs will have an inhibiting effect. First and foremost, the purification of the EVs must be validated for each sample since the lack in specific isolation techniques and the heterogenous nature of EVs complicates the adequate discrimination between small EVs and large EVs (28, 29). Then, the impact of macrophage-derived EVs on mouse OPC cultures is investigated with regard to OPC differentiation.

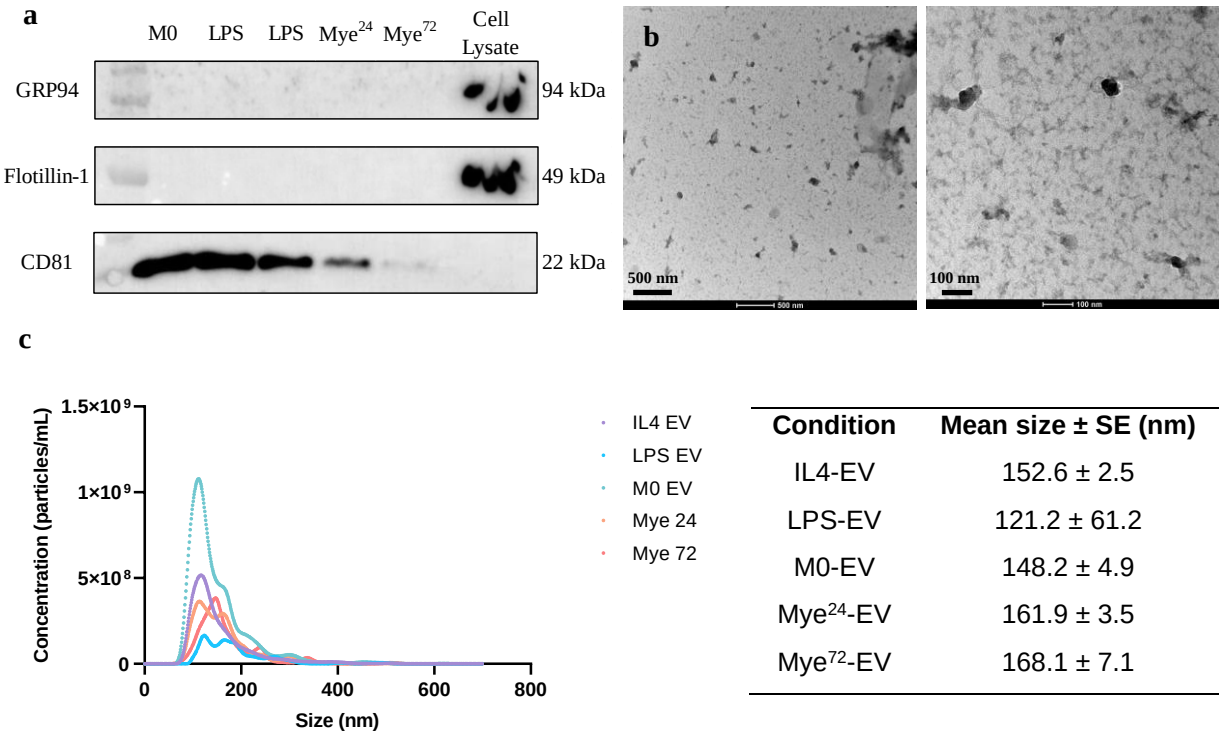
BMDM-Derived EVs Contain Particles That Display EV Characteristics – The purification of EVs derived from BMDMs treated with IL4 (IL4-EV), LPS (LPS-EV), myelin for 24 hours (24h), myelin for 72 hours (72h), and untreated BMDMs (M0-EV) is validated through western blot, nanoparticle tracking analysis (NTA), and TEM. In the western blotting analysis, three categories of general EV markers are measured due to the lack of specific small EV markers: transmembrane lipid-bound EV proteins, integral proteins, and intracellular proteins. Transmembrane lipid-bound EV markers are proteins present on the membranes of EVs, such as CD81. Integral proteins, such as Flotillin-1, are proteins which are present within the EVs and can be detected after EV-lysis. Intracellular proteins should be absent in EVs, hence they are expected to only be detected in cell lysate samples, serving as a positive control, with markers such as GRP94 (30). The western blot results reveal the presence of the transmembrane CD81 marker and the absence of the intracellular GRP94 marker in EV samples. The integral Flotillin-1 marker, however, was not detected (Fig. 2a). Thus, the results partly validate the presence of EVs in each sample.

In TEM, small EVs are characterised through morphological analysis to validate the typical spherical or cup-shaped morphology of the EVs (53). The TEM images reveal a low appearance of spherical shaped particles portraying small EVs (Fig. 2b). In addition to their morphology, the size of the EVs can be observed which corresponds to the size of small EVs, being smaller than 200 nm (37).

Lastly, the size distribution profiles of the EV samples are composed through NTA. The graph and complementary table provide certainty that the correct particles are isolated in terms of size (37) (Fig. 2c). The peak of the graph for all conditions reveals an overall particle size smaller than 200 nm, confirmed by the mean particle size for each condition shown in the table.

Taking into account the presence of the transmembrane CD81 marker along with the accurate size of the particles, strongly suggests that the purification of the small EVs in each sample is sufficiently validated.

Immunofluorescence and Quantitative PCR Analysis Reveals No Significant Impact of BMDM-Derived EVs on OPC Differentiation – In order to determine the impact of the macrophage-derived EVs on OPC differentiation, an immunofluorescence staining is performed. The OPC cultures are stained for MBP and O4 to determine the differentiation status of the oligodendrocytes expressed as MBP/O4. MBP is a marker for mature and differentiated oligodendrocytes, whereas O4 is a marker for immature oligodendrocytes (54). The MBP/O4 ratio of OPCs treated with a specific EV concentration of $4x10^8$ particles/mL compared to EVs from a constant BMDM number of 300 000 shows no significant difference. In fact, the conditions between these 2 groups are quite similar, except for M0-EVs of $4x10^8$ particles/mL which reveal the highest insignificant ratio along with the highest standard deviation (Fig. 3a,b). Mye^{24}-EVs result in a non-significant increase in MBP/O4 compared to the control OPCs. Mye^{72}-EVs cause a non-significant decrease in MBP/O4 compared to the control OPCs. Moreover, mye^{24}-EVs display no significant increase on OPC differentiation compared to mye^{72}-EVs (Fig. 3a,b). IL4-EVs reveal an insignificant higher MBP/O4 compared to control OPCs, likewise, LPS-EVs display an insignificant lower MBP/O4 (Fig. 3a,b). Overall, this indicates that IL4-EVs and mye^{24}-EVs have a similar, non-significant, stimulating impact on OPC differentiation. Correspondingly, LPS-EVs and mye^{72}-EVs have a similar, non-significant,



Condition	Mean size ± SE (nm)
IL4-EV	152.6 ± 2.5
LPS-EV	121.2 ± 61.2
M0-EV	148.2 ± 4.9
Mye^{24}-EV	161.9 ± 3.5
Mye^{72}-EV	168.1 ± 7.1

Fig. 1 – Characterization of small EVs released by bone marrow-derived macrophages with different activation states. **a** Western blot analysis of EV protein markers CD81, Flotillin 1, and GRP94 as positive control marker. The EVs are derived from mye^{24}-macrophages (mye^{24}-EVs), mye^{72}-macrophages (mye^{72}-EVs), IL4-stimulated BMDMs (IL4-EVs), LPS-stimulated BMDMs (LPS-EVs), and non-activated BMDMs (M0-EVs) (EV concentration = 4x10^{8} particles/mL). **b** Representative TEM images of BMDM-derived EV morphology (scale bars: 500 nm and 100 nm). **c** Particle size distribution by NTA of IL4-EVs, LPS-EVs, M0-EVs, mye^{24}-EVs, and mye^{72}-EVs. Respective data is presented as mean ± SE.

inhibiting impact on OPC differentiation. Next, the impact of macrophage-derived EVs on OPC differentiation is also defined on mRNA level by measuring the gene expression of several stage specific differentiation markers through qPCR. *Mbp*, *Cnpase*, *Mog*, *Plp*, *Mag*, and *Sox10* are markers corresponding to mature oligodendrocytes whereas *Pdgfra* and *Nkx2*.2 correspond to immature oligodendrocytes (55). Mye^{24}-EVs show higher, yet non-significant, expression levels of mature oligodendrocyte markers but mye^{72}-EVs do not show higher elevated levels of immature oligodendrocyte markers in comparison to mye^{24}-EVs (Fig. 3c,d). Surprisingly, IL4-EVs express insignificant lower *Mbp* levels than LPS-EVs (Fig. 3e). Altogether, mye^{24}-EVs and mye^{72}-EVs display a non-significant stimulating and inhibiting effect on OPC differentiation, respectively. Additionally, the results of IL4-EV and LPS-EVs contradict the findings of the immunofluorescence analysis.

BMDM-Derived EVs Display No Significant Effect on Lipid Metabolism in OPCs on mRNA Level – Lipid metabolism and transport is increased during OPC differentiation and remyelination considering the importance of lipids for the formation and maintenance of myelin and the maturation of OPCs into heavily branched mature oligodendrocytes (45, 47-49). Therefore, OPC differentiation can be indirectly analysed by measuring the gene expression levels of lipid metabolism markers such as *Abca1*, *Abcg1*, *Lxra*, *Apoe*, and *Scd1* (56). Activation of LXRa results in an upregulation of ABCA1, ABCG1 and SCD1, in turn ABCA1 and ABCG1 promote the cellular efflux of cholesterol to ApoE (9). However, SCD1 is predominantly activated by LXRb (9, 25). Mye^{24}-EVs show higher, yet non-significant,

expression levels of lipid metabolism markers such as ABCA1 and LXRa, indirectly revealing a potential non-significant increase in OPC differentiation (Fig. 4). Overall there is a trend that could be significant with a larger sample size.

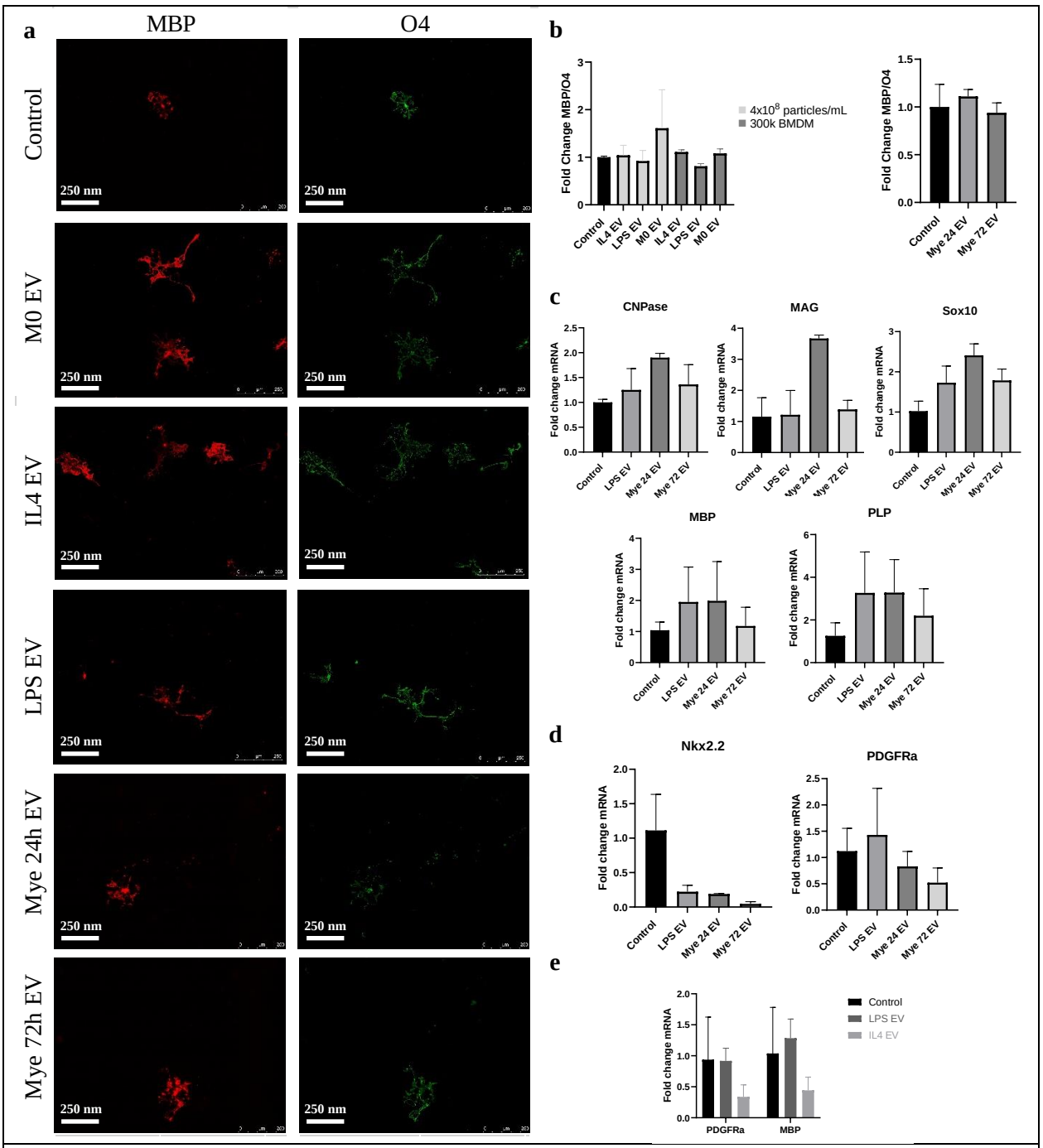


Fig. 3 – Immunofluorescence and quantitative PCR (qPCR) analysis of the impact of EVs derived from bone marrow-derived macrophages with different activation states on oligodendrocyte progenitor cell (OPC) differentiation. **a** Representative images of OPCs treated with EVs derived from IL4-stimulated macrophages (IL4-EV), LPS-stimulated macrophages (LPS-EV), mye^{24}-macrophages (mye^{24}-EV), mye^{72}-macrophages (mye^{72}-EV), non-activated macrophages (M0-EVs), and untreated control OPCs, immunostained against MBP and O4 (scale bar = 250 nm) (EV concentration = 4x10^8 particles/mL) **b** Quantification of MBP/O4 immunofluorescence staining of OPCs treated with LPS-EVs, IL4-EVs, M0-EVs, mye^{24}-EVs, and mye^{72}-EVs. (EV concentration = 4x10^8 particles/mL; or 300 000 BMDMs) (LPS, IL4, M0: N = 3; mye^{24}, mye^{72}: N = 4). **c** qPCR of mature oligodendrocyte markers in OPCs treated with mye^{24}-EVs and mye^{72}-EVs (*Cnpase*, *Sox10*, *Mag*: N = 2; *Mbp*, *Plp*: N = 8). **d** qPCR of immature oligodendrocyte markers in OPCs treated with mye^{24}-EV and mye^{72}-EV (*Nkx2.2*: N = 2; *Pdgfra*: N = 8). **e** qPCR of *Pdgfra* and *Mbp* in OPCs treated with IL4-EVs and LPS-EVs (N = 4). Statistical analysis was performed with the non-parametric Kruskal–Wallis test by ranks and $p<0.05$ was considered statistically significant.

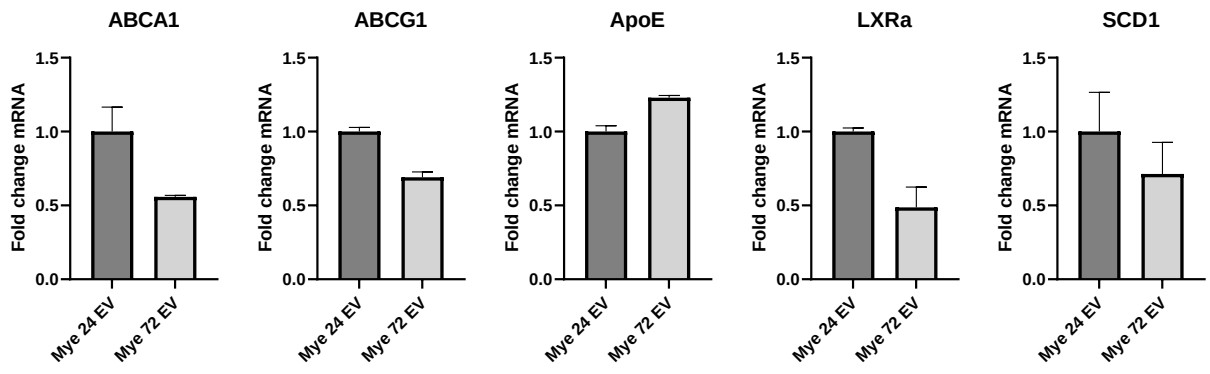


Fig. 4 – Quantitative PCR analysis of the impact of EVs derived from bone marrow-derived macrophages with different activation states on the lipid metabolism of oligodendrocyte progenitor cells (OPC). Gene expression of lipid metabolism markers in OPCs treated with EVs derived from mye^{24}-macrophages and mye^{72}-macrophages (N = 2). Statistical analysis was performed with the non-parametric Kruskal-Wallis test by ranks and $p<0.05$ was considered statistically significant.

DISCUSSION & CONCLUSION

Ample evidence indicates that macrophages and EVs display an ambiguous repertoire in MS pathology. Both have shown to be contributors to tissue damage on one hand as well as mediators of regeneration on the other hand (18, 19, 22, 27). In order to clarify the exact communication mechanism between OPCs and macrophages so the role of macrophages in CNS repair is better understood, we hypothesised that macrophages secrete EVs to mediate their immunomodulatory and regenerative properties. As a result, EVs are produced with either disease-promoting or wound-healing effects depending on the phenotype of the parental macrophage (27, 32, 38, 39).

We aimed to define the impact of EVs derived from macrophages on remyelination in MS. The hypothesis was that lipids within EVs released by macrophages exposed to myelin for short and long term will promote and suppress OPC differentiation, respectively. Likewise, macrophages stimulated with IL4 and LPS secrete EVs with pro-regenerative and pro-inflammatory capacities that promote and suppress OPC differentiation, respectively.

Prior to evaluating the impact of macrophage-derived EVs on OPC differentiation, we characterised the isolated EVs to validate its purity. Van den Broek et al. (2020) characterised microglial-derived EVs prior to experimentation. The western blot showed clear bands for the Flotillin-1 marker and the TEM images presented spherical-shaped EVs that were more abundantly present (53). This is unlike our TEM photos with a relatively low quality caused by a substantial amount of seemingly non-vesicular particles or aggregates. Moreover, our results could not validate the presence of Flotillin-1 through western blot, which could be caused by an inadequate lysis of EVs. In this case, the absence of the GRP94 marker for the EV samples is invalid and the presence of CD81 is the only conclusive western blot result. This raises the question whether other particles within the 200nm size range are able to express CD81. According to Yong et al. (2016), CD81 is expressed by immune cells, hepatocytes, and most stromal and epithelial cells (57). The diameter of epithelial cells ranges from 8-21µm, immune cells range from 7-30µm, hepatocytes range from 25-30µm, and stromal cells range from 17.9-30.4µm (58-61). Hence, the particles in the EV samples expressing CD81 are most likely small EVs. Nevertheless, the integral annexin-A2 can be measured to confirm inadequate EV lysis.

Contrary to our expectations, our results revealed no significant impact of macrophage-derived EVs on OPC differentiation. This impact was evaluated by analysing OPC differentiation with different approaches. Through immunofluorescence analysis MBP/O4 ratio was quantified for each condition. Through qPCR several stage specific differentiation markers were determined which directly correlate with OPC differentiation. Additionally, the gene expression levels of lipid metabolism related markers were defined through qPCR which indirectly correlate with OPC differentiation. With regard to the immunofluorescence analysis, we anticipated a higher MBP/O4 ratio for OPC cultures treated with IL4-EVs and mye^{24}-EVs compared to those treated with LPS-EVs and mye^{72}-EVs. Our results are in contrast with a previous study by Lombardi et al. (2019), in which the effects of microglial-derived EVs on OPC migration, differentiation, and myelination was investigated. Here, the authors induced the pro-regenerative traits of microglia through exposure with immunosuppressive mesenchymal stem cells (MSCs) and IL4. Although much lower EV concentrations were added to the OPC cultures ($2x10^7$ particles/500 µl), the authors were able to significantly enhance OPC differentiation with microglial-derived EVs (16). Worth mentioning is that the pro-myelinating actions of EVs from MSC-treated microglia cells exceeds that of IL4-treated microglia cells. This raises an interest in the effect of EVs from MSC-treated macrophages on OPC differentiation. Also worth noting is that the authors measured OPC differentiation by means of immunofluorescence analysis of the fraction of mature oligodendrocytes compared to total cell count, quantified as MBP/DAPI, instead of MBP/O4. Therefore, it could be of interest to analyse MBP/DAPI and evaluate if this leads to more profound results. A different study by Willis et al. (2020) also contradicted our findings. Here, the authors aimed to determine the effect of young and aged astrocyte-derived EVs on OPC differentiation, and were able to significantly induce OPC differentiation with young astrocyte-derived EVs (44). The immunofluorescence analysis was quantified in a different manner, as MBP/Olig2

instead of MBP/O4, with a large sample size of 7-11. Hence, it could be relevant to repeat the immunofluorescence staining with a larger sample size and by staining immature oligodendrocytes with Olig2.

Concerning the qPCR analysis of several stage specific differentiation markers, we expected higher levels of mature oligodendrocyte related markers (*Mbp*, *Cnpase*, *Mog*, *Plp*, *Mag*, and *Sox10*) for OPCs treated with IL4-EVs and mye^{24}-EVs compared to those treated with LPS-EVs and mye^{72}-EVs. Correspondingly, OPCs treated with LPS-EVs and mye^{72}-EVs were expected to display higher levels of immature oligodendrocyte markers (*Pdgfra* and *Nkx2*.2). Our findings revealed no significant impact on mRNA level which contradicts a study by Osorio-Querejeta et al. (2019). In this study, the authors evaluated the ability of miR-219a-5p enriched EVs to induce OPC differentiation, compared to miR-219a-5p enriched clinically approved drug delivery vectors. They measured the gene expression levels of mature oligodendrocyte related genes (*Cnpase*, *Mbp* ,*Mog*, and *Plp1*) and OPC related genes (*Pdgfra* and *Ng2*) through qPCR. The authors' results show that only the EVs were able to induce OPC differentiation and significantly increased the expression of *Cnpase*, *Mbp* ,*Mog*, and *Plp1* (62). Thus, EVs are able to affect OPC differentiation on mRNA level regarding *Cnpase*, *Mbp* ,*Mog*, and *Plp1*.

As for the qPCR analysis of lipid metabolism related markers (*Abca1*, *Abcg1*, *Lxra*, *Apoe*, and *Scd1*), increased gene expression was anticipated for OPCs treated with mye^{24}-EVs compared to mye^{72}-EVs. Despite a clear trend being visible according to our expectations, the gene expression levels are not significantly increased or decreased. Our findings partially align with a study by Nelissen et al. (2012) in which the function and expression of LXRs and target genes in oligodendrocytes were investigated. Here, OPCs expressed low levels of *Mog*, *Mag*, and *Mbp* which significantly increased after differentiation to mature oligodendrocytes. Additionally, the expression of *Lxrb* and *Apoe* was significantly increased during maturation. However, and in resemblance to our results, no significant difference was detected in the expression of *Lxra*, *Abca1*, and *Abcg1* (46). Thus, the expression level of *Lxra* and *Lxr* target genes depends on the stage of oligodendrocyte maturation which could explain our insignificant results when taking the small sample size into consideration as well.

In conclusion, our study was unable to define a significant impact of macrophage-derived EVs on OPC differentiation. Although anticipated, pro-regenerative macrophage-derived EVs did not significantly stimulate OPC differentiation and pro-inflammatory macrophage-derived EVs did not significantly inhibit OPC differentiation. Nevertheless, there is a trend for the qPCR analysis of lipid metabolism related genes between mye^{24}-EVs and mye^{72}-EVs in which the small sample size should allow for the possibility of a statistical significant effect when a larger sample size is analysed. Another trend is present in the immunofluorescence analysis of OPCs treated with LPS-EVs and IL4-EVs from a constant BMDM amount of 300 000 which could also potentially uncover a statistical significance with a larger sample size. In the future, it could be interesting to investigate the effect of EVs derived from MSC-stimulated macrophages on OPC differentiation. Furthermore, the effects may be more significant when validating our results *ex vivo* with the use of brain slices and lipidomic analysis of the respective EV cargo may uncover interesting findings. For instance, cerebellum, brainstem, or spinal cord slices can be prepared and cultured for 10 days followed by demyelination which can be induced by adding lysophosphatidylcholine to the medium for several hours. After treatment with IL4-EVs, LPS-EVs, mye^{24}-EVs, and mye^{72}-EVs, remyelination can be established through immunofluorescence analysis and TEM. By means of immunofluorescence analysis, the amount of myelin sheath per axon area can be quantified through the expression of MBP to neurofilament proteins which are markers for myelin and axons, respectively. Through TEM, the G-ratio of the axons can be assessed which represents the thickness of the axonal myelin sheaths compared to axonal size, hence it is an index for axonal myelination (63). Thereafter, our findings could be validated with the use of an *in vivo* model, where cuprizone mice receive EV-treatment and brain slices are analysed, as aforementioned, through immunofluorescence analysis and TEM (64, 65). Ultimately, this study will provide more insights into remyelination and the role of macrophages in MS pathology.

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